Exploring Cd phytoextraction with Brassica napus and Nicotiana tabacum: Breeding and selection versus genetic engineering

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Exploring Cadmium Phytoextraction with *Brassica napus* and *Nicotiana tabacum*: Breeding and Selection versus Genetic Engineering

The research presented in this thesis was carried out at the Vrije Universiteit-Amsterdam in the Netherlands.

Front Cover: Transformed tobacco in the PK2 greenhouse of the Plant Genetics group at the Vrije Universiteit-Amsterdam. Photograph by the author.

Back Cover: Young *Brassica napus* plants, growing on hydroponics in the greenhouse of the Hortus at the Vrije Universiteit-Amsterdam. Photograph by the author.

VRIJE UNIVERSITEIT

Exploring Cadmium Phytoextraction with *Brassica napus* and *Nicotiana tabacum*: Breeding and Selection versus Genetic Engineering

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geboren te Linne

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Chapter 1

General Introduction

Veerle M.J. Grispen, Jos A.C. Verkleij and Henk Schat

Prologue

Metal enrichment of the environment is widely spread, originating both from natural and anthropogenic sources. Natural sources include parent rock and atmospheric emissions from volcanoes and forest fires (Nriagu 1980, 1989; WHO 1992). Compared to essential metals like zinc (Zn), copper (Cu) and iron (Fe), cadmium (Cd) is a non-essential element with no known biological function in animals, plants and fungi. Cd is very toxic, and contamination of agricultural soils is a world wide environmental and health concern. Anthropogenic Cd contamination in soils results mainly from lead (Pb) and Zn mining and especially smelting operations (Kabata-Pendias and Pendias 2001). Smelting activities in the past have lead to high atmospheric Cd loads which in turn resulted in a widespread and diffuse contamination. Numerous are the other ways in which Cd enters our environment. Examples in this wide spectrum range from batteries that contain Cd, industrial waste from electroplating, alloy preparation, household appliances but Cd is also used for luminescent dials in photography and airplane parts (Adriano 2001). Also phosphate fertilizers, sludge and several biocides used in conventional agriculture are known as important sources of Cd (Williams and David 1973; Andersson and Hahlin 1981; Liphadzi and Kirkham 2006). Table 1 lists examples of elevated total soil Cd concentrations as a result of different anthropogenic sources.

Table 1. Enhanced total Cd concentrations in soils due to various anthropogenic activities

Cd source	Total Cd (mg kg^{-1})	Reference
Smelter emission	5; 18; 36	(this thesis), (Mench et al., 1994; Zheljazkov et al., 2008)
Irrigation water	40; 48	(Quartacci et al., 2006; Gupta et al., 2008)
Pb, Cd & Zn mine spoil	92; 155-300; 1051	(Hamon et al., 2002; Milton et al., 2004; Yanqun et al., 2005)
Sewage sludge	2; 42	(Lombi et al., 2003; Wahla and Kirkham 2008)

Soil phytoremediation entails two distinct techniques to either stabilize or extract soil metals and metalloids with the help of plants, called phytostabilization and phytoextraction, respectively. Two approaches for the

development of commercial phytoextraction technologies are believed to have significant promise (Chaney et al., 2007): 1. domesticate natural element (hyper)accumulator plants; 2. clone all genes needed for (hyper)accumulation and (hyper)tolerance and express them in a high-biomass yielding transgenic (hyper)accumulator. *Brassica napus* L. is a high biomass producing crop and interesting as a potential phytoextraction crop for several reasons: first, its close relative *Brassica juncea* has been shown to have potential in phytoremediation (Zhu et al., 1999; Bennett et al., 2003; Lindblom et al., 2006; Navaza et al., 2006); second, it is among the oldest oil-producing crops in Europe and its seeds have the potential for use in biodiesel production.

Plants that are intended to be used for phytoextraction need to be both tolerant and able to accumulate the metals in their above ground parts. This depends on metal availability in the rhizosphere, root uptake, symplastic mobility and xylem loading, and also detoxification and storage inside the shoot (Clemens et al., 2002).

This thesis describes experiments aimed at improving the transport of metals and metalloids, particularly Cd and to a lesser extent Zn and arsenic, to the shoot of *B. napus* L. and *Nicotiana tabacum* L., for phyto-extraction purposes, using classical breeding and selection, as well as genetic engineering.

Metal availability and mobilization in the rhizosphere

Cd was discovered in 1877 as a constituent of Zn carbonate, and mostly co-occurs with Zn and Pb in ores. Cd concentrations in the earth's crust vary from 0.001 - 90 mg/kg (Page 1981; Kabata-Pendias and Pendias 2001). Areas that are not prone to anthropogenic pollution generally fall into the 0.1 - 2 mg/kg range (McLaughlin et al., 1999). In soils Cd occurs mainly in the divalent form. During weathering Cd goes readily in solution and may form complex ions ($CdCl^+$, $CdOH^+$, $CdCl_3^-$), or bind to soluble organic ligands, which can facilitate the uptake into the root; it can also bind to unsoluble organic matter and clay minerals. The same holds for Zn, as they are physically and chemically very alike. The most important factors controlling Cd and Zn ion mobility are pH and redox potential. Both metals are most mobile in acidic

soils within the pH 4.5 to 5.5 range whereas in alkaline soil they are rather immobile (Kabata-Pendias and Pendias 2001). Symbiosis with mycorrhizal fungi can reduce, but also enhance the Zn and Cd uptake rate of the host plant (Ernst et al., 1992). Furthermore, Zn/Cd ratios play an important role in Cd uptake. Zn has an antagonistic effect on Cd accumulation but this effect is Zn and Cd concentration dependant.

Root Metal Uptake

It is assumed that uptake of non-essential elements, like Cd, takes place via transporters of essential elements, i.e. Zn, Fe and calcium (Ca), due to incomplete specificity. Members of the ZIP (Zn regulated transporter/Fe regulated transporter-like protein) gene family, have been shown to transport divalent heavy metal cations (Eide et al., 1996; Grotz et al., 1998; Korshunova et al., 1999; Guerinot 2000; Thomine et al., 2000; Connolly et al., 2002). In the ZIP family of metal transporters, IRT1 was the first protein shown to contribute to uptake of Zn, Mn, Co and Cd when expressed in *Saccharomyces cerevisiae* (Korshunova et al., 1999). Afterwards, in *Arabidopsis thaliana*, IRT1 was shown to represent the main Fe uptake system in root cells (Vert et al., 2002) and when over-expressed it increased Zn and Cd accumulation in root tissue under Fe deplete conditions (Connolly et al., 2002). A general finding among different plant species has been that under Fe limitation the Fe uptake systems are up-regulated which allows Cd to enter root cells (Cohen et al., 1998). ZIP1, 2 and 3 are Zn-regulated transporters and, when expressed in yeast, their Zn uptake activity is partially blocked by Cd (Grotz et al., 1998). In *Thlaspi caerulescens* low-affinity Cd uptake happens alongside with high-affinity Zn uptake mediated by ZNT1, which is the homologue of AtZIP4 (Pence et al., 2000). Another gene family suggested to be involved in Cd entry into plant cells, is the NRAMP family. Initial work in *S. cerevisiae* on AtNRAMP 1,3 and 4 showed that they established Cd uptake and ectopic homologous over-expression of AtNRAMP3 resulted in Cd hypersensitivity (Thomine et al., 2000). However, NRAMP3 appeared to be localized at the tonoplast, thus it is more likely to be involved in intra-cellular metal

mobilization in stead of metal uptake (Thomine et al., 2003). Cd is chemically very similar Ca. This similarity is demonstrated by the ability of Cd to compete with Ca for binding to calmoduline (Rivetta et al., 1997). With respect to Cd hitchhiking along with Ca, it was shown that the LCT1 protein from wheat mediates Ca influx and renders yeast cells Cd hypersensitive because of the LCT1 dependent Cd influx (Clemens et al., 1998). When overexpressed in tobacco, however, LCT1 appears to provide Ca-dependent protection against Cd toxicity (Antosiewicz and Hennig 2004).

Intracellular mobility and vacuolar transport in roots

Once inside the cytosol Cd ions are chelated by either free glutathione (GSH) or phytochelatins (PCs) which are derived from GSH by the action of a γ-glutamylcysteinyl transpeptidase, phytochelatin-synthase (PCS) (Grill et al., 1989; Zenk 1996; Rauser 1999). PCS is a constitutive enzyme requiring post-translational activation by heavy metal/metalloid ions (Grill et al., 1989). In plants PC-based detoxification seems essential for basic tolerance to Cd. Deficiency in PC production in Arabidopsis mutants, either through decreased GSH synthesis, or through mutation of the *PCS* gene itself, results in reduced Cd accumulation and tolerance (Howden and Cobbett 1992). Conversely, PCS expression in cells that normally do not form these peptides (e.g. *S. cerevisiae*) leads to an increase in Cd accumulation (Clemens et al., 1999). Cd and arsenite (As^{3+}) are the most potent activators of phytochelatin synthase. Pb, Zn, Cu, antimony (Sb), silver (Ag), and mercury (Hg) also induce PC synthesis, but to a lower degree. However over-expression of the *AtPCS1* gene in tobacco leads to increased Cd sensitivity (Wojas et al., 2008), possibly due to GSH depletion (Rauser et al., 1991). In general, PCS over-expression does not seem to be the best strategy to enhance Cd accumulation and tolerance (Li et al., 2005; Wojas et al., 2008), because PCS apparently exerts insufficient control over the upstream GSH synthetic pathway. In line with this, when combined with exogenous GSH supply, PCS over-expression does significantly enhance Cd accumulation and tolerance in tobacco (Pomponi et al., 2006). The mere binding of Cd to PCs does not seem to be sufficient

for for detoxification. In *Saccharomyces pombe* the transport of the PC-Cd complex into the vacuole, mediated by the ABC-type transporter, Hmt1, and its subsequent stabilization through incorporation of sulfide leading to high-molecular-weight aggregates (PC-Cd-S) are both essential for normal Cd tolerance. *S. pombe* cells lacking the tonoplast transporter Hmt1 are just as Cd sensitive as a *pcs* knock-out strain (Ortiz et al., 1992; Ortiz et al., 1995; Clemens 2006). Also in plants PC-Cd complexes are mainly found in vacuoles (Vögeli-Lange and Wagner 1996). The responsible transporter has not been identified yet, but it has the characteristics of an ABC-type transporter (Salt and Rauser 1995). However, plant PC-Cd complexes contain only little acid-labile sulfide, as compared with *S. pombe* (de Knecht et al., 1994). Vacuolar transport of non-PC-bound Cd has been disputed, because of the absence of free ionic Cd at considerable concentrations in the cytosol (Clemens, 2006). However, ectopic homologous over-expression of several members of the CAX family of vacuolar cation proton exchangers, AtCAX2 and AtCAX4, results in enhanced Cd accumulation in the root (Koren'kov et al., 2007). Other potential vacuolar Cd transporters are members of the CDF cation diffusion facilitator family, recently renamed MTP. AtMTP1 (synonymous with ZAT) is involved in the vacuolar sequestration of Zn in Arabidopsis. Homologous ectopic over-expression enhances Zn tolerance and Zn accumulation in the roots (van der Zaal et al., 1999). Other members are involved in vacuolar Mn accumulation and Mn tolerance (Delhaize et al., 2003), and TgMTP1 from the Ni hyperaccumulator, *Thlaspi goesingense*, has been suggested to be involved in Ni tolerance (Kim et al., 2004). However, to date there is no clear-cut evidence of CDF-mediated vacuolar Cd transport *in planta*. Another potential vacuolar Cd transporter is the 1b P-type ATPase, HMA3, a vacuolar member of the heavy metal transporting ATPase family (Gravot et al., 2004), however, evidence of *in planta* HMA3-driven Cd transport is not available.

Next to PCs, metallothioneins (MTs) are abundant metal compounds in plants. In contrast to PC-metal complexes, MT-metal complexes are not transported into the vacuole. MTs are often strongly expressed in Zn/Cd hyperaccumulators such as *T. caerulescens* (Roosens et al., 2004; van de Mortel et al., 2006),

suggesting a possible role for them in the hyperaccumulation phenotype. Higher plant metallothioneins are classified into four types, of which 1, 2 and 3 are believed to function in Cu homeostasis and tolerance, because their expression is regulated primarily by Cu and barely or not by other metals (Murphy and Taiz 1995; van Hoof et al., 2001; Guo et al., 2003; Roosens et al., 2004; Guo et al., 2008). Type 4 is involved in Zn homeostasis (Lane et al., 1987; Guo et al., 2008). Nevertheless, all of them can bind a variety of metal and metalloid ions, including non-essential ones (Cobbett and Goldsbrough 2002). Therefore, plant MTs have been suggested to function in non-essential metal detoxification, next to essential metal homeostasis (Merrifield et al., 2004; Merrifield et al., 2006; Guo et al., 2008).

Xylem and phloem loading

Once in the stelar tissues, metals can be loaded into the xylem or phloem. Active Zn and Cd xylem loading is mediated by members of the subfamily of heavy metal transporting 1b P-type ATPases (HMAs) (Axelsen and Palmgren 2001). The HMA family is subdivided into two groups, Cu/Ag transporting pumps and Zn/Cd/Co/Pb-transporting pumps. Plants express both types. HMA1-4 in *A. thaliana* belong to the latter, where AtHMA2 and 4 are important for Zn translocation. Both are localized at the plasma membrane of xylem parenchyma, particularly in the root, where they efflux Zn from the parenchyma cells into the xylem vessels. The double mutant *hma2hma4* suffers from inadequate Zn supply to the leaves (stunted growth, chlorosis), and Zn supplementation is enough to complement this mutant (Hussain et al., 2004). Recently it has been shown that the same transporters are responsible for virtually all of the Cd translocation to the shoot in *A. thaliana* (Wong and Cobbett 2009). When expressed in *S. cerevisiae*, AtHMA4 complements the Cd hypersensitive mutant *ycf1* of *S. cerevisiae* (Mills et al., 2005). HMA4 is strongly expressed in Cd/Zn hyperaccumulators (van de Mortel et al., 2006; Hanikenne et al., 2008), and RNAi-mediated silencing in *A. halleri* leads to reduced translocation to the shoot, as well as to significant losses of Zn and Cd tolerance, probably through metal accumulation in the root (Hanikenne et

al., 2008).

PCs might also play a significant role in long distance Cd transport in plants, although they are usually not found at detectable concentrations in xylem sap. From grafting experiments and organ-specific PCS expression in Arabidopsis *pcs* mutants it appears that PCs and Cd are transported via the phloem at significant rates, in both directions (Gong et al., 2003; Chen et al., 2006). In *B. juncea* the Cd concentration in phloem sap is even much higher than in xylem sap (Mendoza-Cózatl et al., 2008).

Detoxification and storage in the shoot

Metals usually reach the apoplast of leaves in the xylem sap, from where are scavenged by leaf cells (Marschner 1995). The responsible transporters are unknown, but there are obvious candidates, belonging to the same families that are involved in root uptake (see above). The same holds for vacuolar transport, which mainly takes place in the epidermal cell layers and trichomes, at least in case of Cd (Salt et al., 1995).

Outline of this thesis

The start of my thesis is focused on phytoremediation of moderate contaminated soils in the "Nederlandse en Belgische Kempen". Chapter 2 describes the evaluation of *Brassica napus* L. accessions as possible candidates for phytoremediation, based on hydroponics and field studies. The *B. napus* accessions that performed best in the field based on two important parameters (shoot metal accumulation and metal translocation rate from root to shoot) were then crossed with each other and tested on polluted soil from the "Kempen" in a greenhouse experiment and results are described in chapter 3. Because classical selection of *Brassica* cultivars does not seem to lead to better results, at least not in the near future, the further study has been focused on the second approach, that is, to improve Cd-phytoextraction via genetic modification. A number of genes were selected, which seem to play an important role in uptake and transport of heavy metals. Because of the slow regeneration process and the low success rate of transformation of *B. napus*

we switched to *Nicotiana tabacum* SR1, which is easier to transform. The first results of *AtMT2b* expression in *N. tabacum* when exposed to arsenite are described in chapter 4. In chapter 5 the *AtMT2b* expressing tobacco has been re-transformed with *AtHMA4*, double transformants were then tested for their Cd and Zn tolerance and accumulation. Finally, the main results of the described experiments are discussed in chapter 6, the general discussion.

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Chapter 2

Phytoextraction with *Brassica napus* L.: a Tool for Sustainable Management of Heavy Metal Contaminated Soils

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Abstract

Phytoextraction is a promising tool to extract metals from contaminated soils and *Brassica napus* L. seems to be a possible candidate species for this purpose. To select accessions with the ability to accumulate cadmium, hydroponically grown 21 day old seedlings of 77 *B. napus* accessions were exposed to 0.2 µM $CdSO_4$ for an additional 10 days. The effects of Cd on several parameters were quantified i.e.; shoot Cd concentration ([Cd]shoot), total amount of Cd in shoots (Total Cd) and the shoot to root Cd concentration ratio (S/R ratio). Though generally natural variation was low for [Cd]shoot, Total Cd and S/R ratio, a number of accessions could be selected. Our results indicated that Total Cd and S/R ratio are independent parameters for Cd accumulation and translocation. The selected varieties were then tested in field experiments on two locations nearby metal smelters. The two locations differed in extractable soil Cd, Zn, Ca concentration and pH levels. On both locations *B. napus* accessions showed significant differences in [Cd]shoot and Total Cd. Furthermore we found significant correlations between Cd and Zn accumulation in shoots. There were site-specific effects with respect to Cd accumulation in the *B. napus* accessions, however, two accessions seem to perform equally well on both sites. The results of the field experiment suggest that certain *B. napus* accessions are suitable for phytoextraction of moderately heavy metal contaminated soils.

Introduction

Natural occurring processes (volcanism, erosion) but in particular human activities contribute to heavy metals being released in the environment. Cadmium (Cd) is a widespread heavy metal, released into the environment by heating systems, metallurgic industries, waste incinerators, urban traffic, cement factories and as a contaminant of phosphate fertilizers (Sanita di Toppi and Gabbrielli, 1999). Contamination of agricultural soils by Cd is a world wide environmental and health wise concern. For agricultural areas most part of the yearly input of Cd is due to the application of phosphorus fertilizers. Soils are often heavily contaminated with both Cd and zinc
(Zn), due to similar geochemical behaviour of these metals (Narwal et al., 1993). In the Netherlands the landfill of Cd containing materials is higher compared to other countries in the
EU. This landfill consists of household waste and jarosite from the Dutch zinc processing industries. The atmospheric load in particular is somewhat higher than the average in the EU. The higher atmospheric load is due to a high inflow of Cd into waste incinerators, via household waste, and due to transboundary pollution from neighboring countries (Guinee et al., 1997). This study will discuss in particular the situation for the area in the mid south of the Netherlands, at the border with Belgium. Since the last century the environment in the Dutch and Belgium ‘‘Kempen’’ has been severely contaminated as a result of high emissions of heavy metals by the
metal processing industry. In an area of approximately 450 km2, high concentrations of heavy metals are found, especially Cd and Zn, caused by atmospheric deposition around the source, of which a large part is designated as agricultural area. Most conventional remediation approaches
(e.g. excavation and land filling) are costly and extremely environmentally disruptive. Moreover, availability of good replacement soil for backfilling is limited. An alternative technology available to clean up a variety of organic and inorganic pollutants is phytoremediation which uses plants and their associated microbes for environmental clean up (Raskin et al., 1994; Salt

et al., 1995a, 1998). For phytoremediation the ideal plant should possess multiple traits like fast growing, have high biomass, deep roots, be easy to harvest and should tolerate and accumulate a range of heavy metals in their aerial and harvestable parts (Clemens et al., 2002). We are interested in the possibilities of *B. napus* (rapeseed) as a candidate phytoextraction crop for several reasons. First of all rapeseed is among the oldest cultivated oil-producing plants; in Europe it is cultivated since the 14th century. Today, rapeseed varieties are mainly used in food applications, but to a growing extent also in the production of biofuel. Phytoextraction with *B. napus* has the potential to become a profitable enterprise when combined with biofuel production, especially in view of the increasing oil prizes over the coming years. Additionally, its relatedness to Arabidopsis thaliana (Lagercrantz, 1998; Paterson et al., 2001) and Thlaspi cearulescens will enable this study to profit from the knowledge gained on (molecular) processes and mechanisms on metal uptake in these species. Also, its higher biomass production compared to natural metal (hyper) accumulators, contributes to the suitability of *B. napus* as a phytoextraction species. We tested 77 *B. napus* accessions from all over the world under controlled conditions in the greenhouse to determine their Cd extraction potential. From this study a selection of 18 accessions was tested accordingly in the field for their Cd and Zn extraction potential.

Material and Methods

Greenhouse experiment

77 winter oilseed rape accessions of *B. napus* (further details in supplementary table 1) were grown under standard conditions in the greenhouse (22/16 °C day/night; daylight and additional irradiance of PAR = 175 µmol m^{-2} s^{-1} at plant level; 16 h d^{-1}). After germination on garden soil, the seedlings were grown on garden soil for 14 days after which they were transferred to hydroponics. Each accession had three replicates with one seedling per 1-L polyethylene pot. Pots were filled with modified quarter strength Hoagland's solution: 1.5 mM KNO_3, 1 mM Ca $(NO_3)_2$, 0.5 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4$, 0.5 µM

KCl, 12.5 µM H_3BO_3, 1µM $ZnSO_4$, 1µM $MnSO_4$, 0.05 µM $CuSO_4$, 0.05 µM $(NH_4)_6Mo_7O_{24}$, 10µM Fe(Na)EDTA. The pH buffer MES was added at a 2-mM concentration and the pH was set at 5.5 using KOH.

After the seedlings had grown for two weeks on nutrient solution, they were exposed for ten days to 0.2 µM $CdSO_4$. The solutions were refreshed twice a week. Before harvest the roots were desorbed with ice-cold 5-mM $PbNO_3$ for 1 h. Several parameters were determined: [Cd]shoot, S/R ratio and Total Cd. In order to calculate the total uptake of Cd in the shoots of a plant (Total Cd), shoot Cd concentration was multiplied by the shoot dry weight. Nine accessions with a high Total Cd and nine accessions with a high S/R ratio were selected for the field trial.

Field experiment

Plant material to be transferred to the field

Seeds of the 18 selected accessions were germinated on garden soil and grown for the first 14 days in the greenhouse on garden soil at a temperature of 15 °C without supplementary light. After 14 days the plants were transplanted to individual pots filled with garden soil and kept in the greenhouse for another 14 days after which they were put at 5 °C for five weeks in order to vernalize. Early May 2004 the winter accessions were transplanted to the field.

Experimental design

The field study was conducted at two independent sites in the proximity of a metal smelter, one situated in Belgium (former maize field near Umicore, Balen) and one in the Netherlands (near Budelco, Budel). Both sites have been contaminated by atmospheric deposition of Cd, Zn and Pb. The field experiment in Budel comprised three plots (15 m^2 per plot) for the *B. napus* accessions with a total area of 45 m^2. Each plot was divided in 15 subplots of one m^2 with four plants per subplot, giving room to 60 plants per plot,

totalling 180 plants per location. This allowed for ten replicates for each of the 18 *B. napus* accessions. The accessions were randomly assigned to the subplots. There was no fertilization applied to the plots and weeds were removed by harrowing. The experimental design in Balen was according to the experiment in Budel. Above ground parts of the *B. napus* accessions were harvested and bulked end of August, respectively.

Soil analysis

At the time of plant harvest, four soil samples were taken for analysis in every plot at a depth of 15-30 cm. The samples were air-dried over night at 70 °C. For the analysis of total metal concentrations in the soil, approximately 1 g was wet-ashed in 2 ml of 37%HCl/65%HNO_3 (1/4 v/v) in Teflon bombs at 140 °C for 7 h. The concentrations of Cd, Zn, Pb and Ca were determined using flame atomic absorption spectrophotometry (AAS) (Perkin Elmer 1100B). Extractable metal concentrations were measured after shaking 10 g of soil with either 25 ml distilled water or 1 M NH_4Cl, for 2 h at room temperature. The extracts were centrifuged at 2500 rpm for 15 min and the supernatants were filtered (Glass micro fibre filter, Whatman) and analysed for Cd, Zn, Pb and Ca using flame AAS. Soil pH was determined in a 1:2.5 (w/w) soil to distilled water mixture.

Plant analysis

The root system of individual plants grown on hydroponics in the greenhouse, was desorbed with ice-cold 5-mM $PbNO_3$ for 30 min. Afterwards the roots were rinsed with distilled water and shoot and root were separated. The plant samples were dried at 70 °C for 48 h., after which dry weight of shoots and roots was determined. Approximately 100 mg of dried plant material was wet-ashed as described above.

After DW estimation of the shoots of the plants from the field experiment, the

whole sample was shredded and mixed in a garden shredder. For each of the 18 accessions from both locations, four sub samples were taken (n=4) to be finely ground in a blender after which approximately 100 mg of dried plant material was wet-ashed as described before. On our "Balen" location two accessions died (nr 56 & 68), leaving us with 16 accessions, each with four sub samples (n=4). Concentrations of Zn and Cd were determined using AAS and by graphite furnace (Perkin Elmer 4100 ZL), respectively.

Statistical data analysis

Differences in Cd and Zn accumulation between the different *B. napus* accessions were tested for statistical significance using one-way ANOVA, followed by a Tukey's test for comparison of individual means, using the statistical program SPSS 10.1 for Windows (SPSS Inc. 2000). In some cases a non-parametric analysis of variance, the Kruskal Wallis Test, was performed.

Results

Greenhouse experiment

Metal accumulation in shoots

In order to assess the Cd accumulation trait, 3 parameters were determined, [Cd]shoot, Total Cd and S/R ratio. There was a clear natural variation in Cd accumulation (Fig. 1a and 1b). [Cd]shoot ranged from 9 to 25 mg g^{-1} DW where Total Cd showed a variation ranging from 7 to 21 mg $plant^{-1}$ DW. Plant biomass production varied from an average of 470 to 1830 mg $plant^{-1}$ DW (data not shown). The third parameter used for assessing the Cd accumulation trait showed the highest variation; the S/R ratio ranged from 0.6 to 0.1 within the accessions (Fig. 1c). However, only one accession exhibited the highest S/R ratio (0.60). All other accessions exhibited a S/R ratio <0.35. According to Total Cd in shoots (Table 1a) and the S/R ratio (Table 1b), 18 *B. napus*

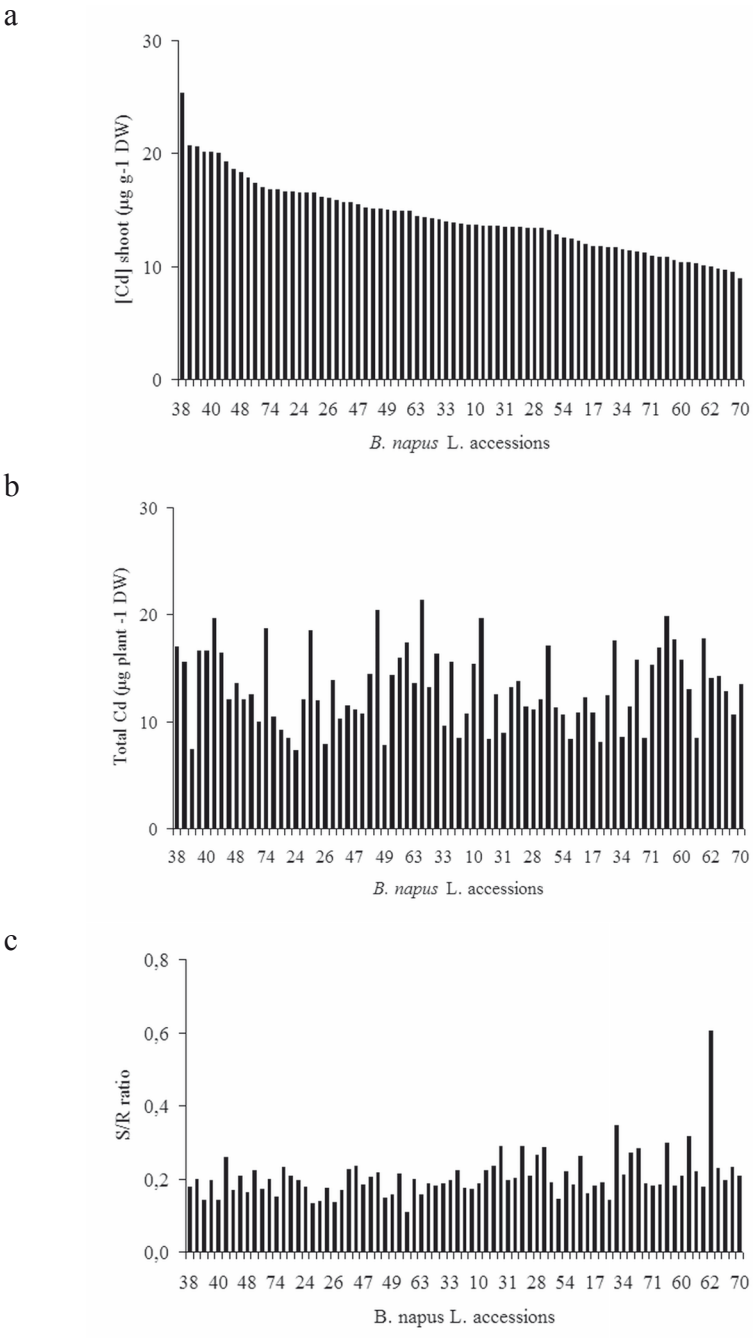

Figure 1. (a) [Cd] shoot (µg g-1 DW) for the B. napus L. accessions (n=3) tested on hydroponics. (b) Total Cd (µg plant-1 DW) for the B. napus L. accessions (n=3) tested on hydroponics. (c) S/R ratio for the B. napus L. accessions (n=3) tested on hydroponics.

accessions were selected for the field experiment based on the highest values (9 with highest total Cd values and 9 with the best S/R ratio). None of the 18 accessions could be selected on both high Total Cd and high S/R ratio.

Table 1a Total Cd in shoots (µg plant^{-1} DW) of *B. napus* L. accessions selected on Cd accumulation for the field experiment (mean ± s.e., n=3).

Accession nr.	Total Cd	s.e.
16	20.4	1.7
73	19.8	1.4
56	19.6	2.1
22	18.5	0.7
68	17.7	1.1
5	16.3	1.1
60	15.7	1.6
30	8.1	0.3
24	7.4	0.2

Table 1b S/R ratio (Cd concentration; µg g^{-1}DW) of *B. napus* L. accessions selected on Cd translocation for the field experiment (mean ± s.e., n=3).

Accession nr.	S/R ratio	s.e.
62	0.60	0.09
69	0.35	0.06
67	0.32	0.06
55	0.29	0.06
64	0.27	0.05
44	0.26	0.02
20	0.26	0.01
40	0.14	0.00
11	0.13	0.00

Field experiment

Soil analysis

The Total Cd concentration in the soil differed significantly between the sites (Table 2). The total soil Cd concentrations were 2.5 and 5.5 mg kg^{-1} soil DW for Budel and Balen respectively. The total fractions of Zn and Pb were higher in the Balen soil. Although the water extractable fractions did not differ greatly between both sites, water extractable Zn was higher in the Budel soil. Water extractable Ca concentrations were higher for the Balen soil, as was the soil pH (4.2 versus 5.7).

Table 2 Soil characteristics (15-30 cm) of the field plots in Budel and Balen, metals (mg kg^{-1} soil DW), Ca (mg kg^{-1} soil DW) and pH (H_2O), (mean ± s.e.).

		Budel (n=4)		Balen (n=4)	
37% HCl/65% HNO_3	Cd	2,5	±0,3	5,5	±0,3
(¼ v/v)	Zn	106	±11	390	±72
total soil	Pb	30	±3	167	±5
	Ca	229	±93	1282	±82
1M NH_4Cl	Cd	1,4	±0,1	3,1	±0,1
extractable soil	Zn	72	±7	43	±6
	Pb	6,6	±0,3	1,0	±0,1
	Ca	36	±7	134	±9
H_2O	Cd	0,2	±0,01	0,22	±0,01
extractable soil	Zn	3,8	±0,5	2,1	±0,4
	Pb	0,2	±0,1	0,8	±0,3
	Ca	18,1	±1	28,3	±4
pH (H_2O)		4,2	±0,1	5,7	±0,1

Metal accumulation in shoots

The capacity for Cd accumulation was determined for the different field grown accessions. [Cd]shoot were determined after a 4 month growing period (Fig. 2a). At location Balen we found a variation range for [Cd]shoot of 3.6 to 8.1 mg kg^{-1} DW, with an average of 5.6 mg kg^{-1} DW. At location Budel this variation ranged from 5.2 to 11.3 mg kg^{-1} DW, with an average of 7.6 mg kg^{-1} DW. Although total extractable Cd at location Balen was approximately more than double the amount found at location Budel (Table 2), the plants grown in Budel showed higher [Cd]shoot. The plants with a high Cd uptake also appeared to exhibit enhanced Zn uptake. In Balen, [Zn]shoot ranged from 342 to 1215 mg g^{-1} DW with an average of 657, In Budel [Zn]shoot showed a smaller variation, 743 to 1414 mg g^{-1} DW with an average of 1151 (Fig. 2b).

At both locations, accessions showed significant differences concerning [Cd]shoot and [Zn]shoot. Searching for possible correlations between Cd and Zn concentrations in the shoot and total Cd and Zn uptake in shoots we depicted the results of all accessions together in fig. 3 and fig. 5. In Budel (Fig. 3a and fig. 5a) all 18 accessions survived, and with 4 sub samples resulted in n=72. Whereas in Balen (Fig. 3b and fig. 5b) 2 accessions died, resulting in an n-value of 64. A clear and significant correlation (at 0.01 level) was observed between the [Cd]shoot and [Zn]shoot in the accessions at both locations (Fig. 3a,b). Comparing the two different locations there was hardly any correlation found with respect to [Cd]shoot and [Zn]shoot. By taking plant biomass into account, Total Cd and total Zn could be determined. The 16 accessions (with n=4) in Balen accumulated an average of 979 mg Cd $plant^{-1}$ within a range of 274 to 1712 mg Cd $plant^{-1}$. In Budel, the 18 accessions showed (with n=4) an average of 1037 mg Cd $plant^{-1}$ with a variation range of 416 to 1621 mg Cd $plant^{-1}$ (Fig. 4a). The accessions in Balen accumulated an average of 105 g Zn $plant^{-1}$ within a range of 10 to 336 g Zn $plant^{-1}$. In Budel the accessions showed and average of 150 g Zn $plant^{-1}$ within a range of 33 to 596 g Zn $plant^{-1}$ (Fig. 4b). A significant correlation was found between total amounts of Cd and Zn in the shoots at both locations (Fig. 5a and fig. 5b). Two accessions

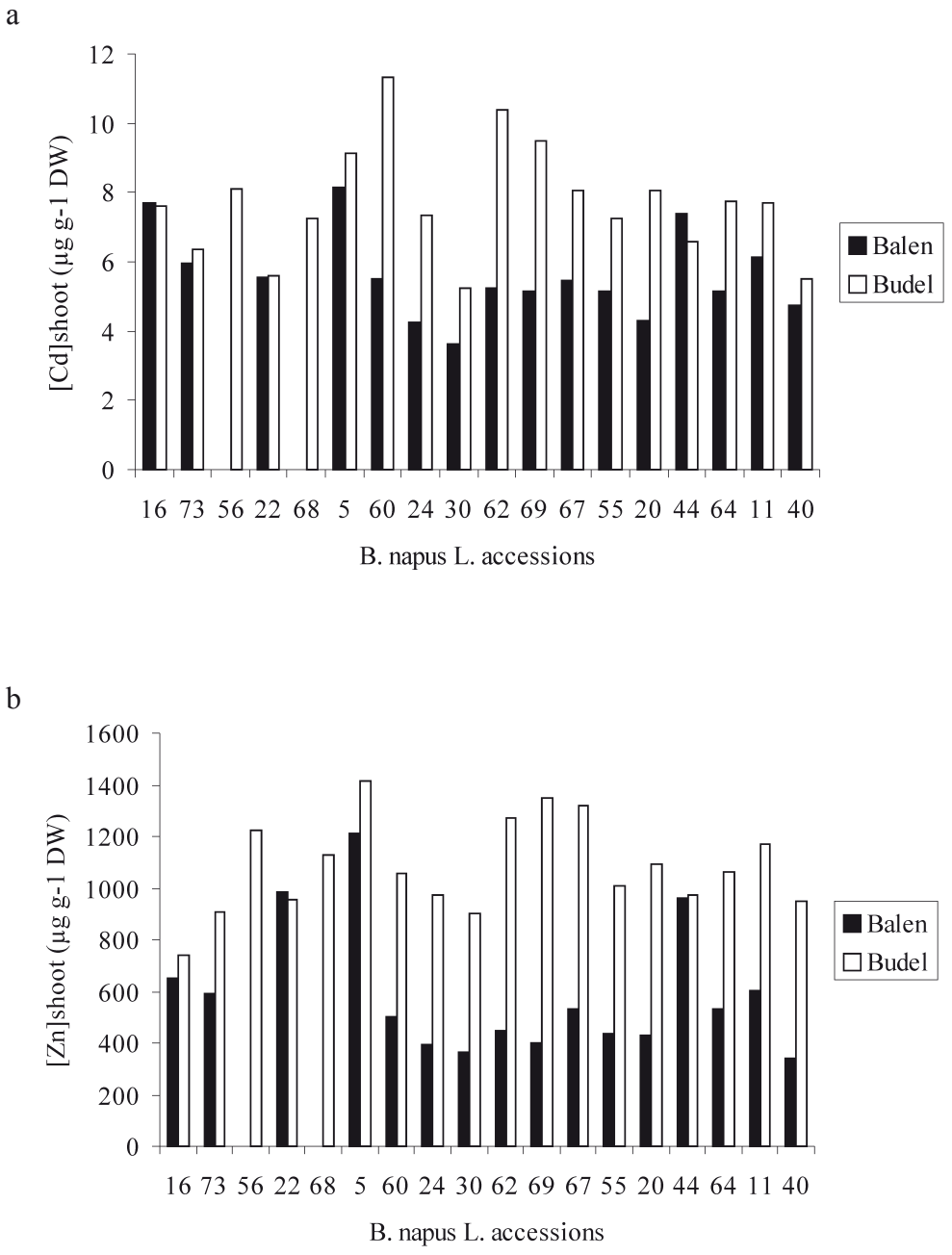

Figure 2. (a) [Cd]shoot (µg g-1 DW) of the B. napus L. accessions (n=4) in Budel and Balen. (b) [Zn]shoot (µg g-1 DW) of the B. napus L. accessions (n=4) in Budel and Balen.

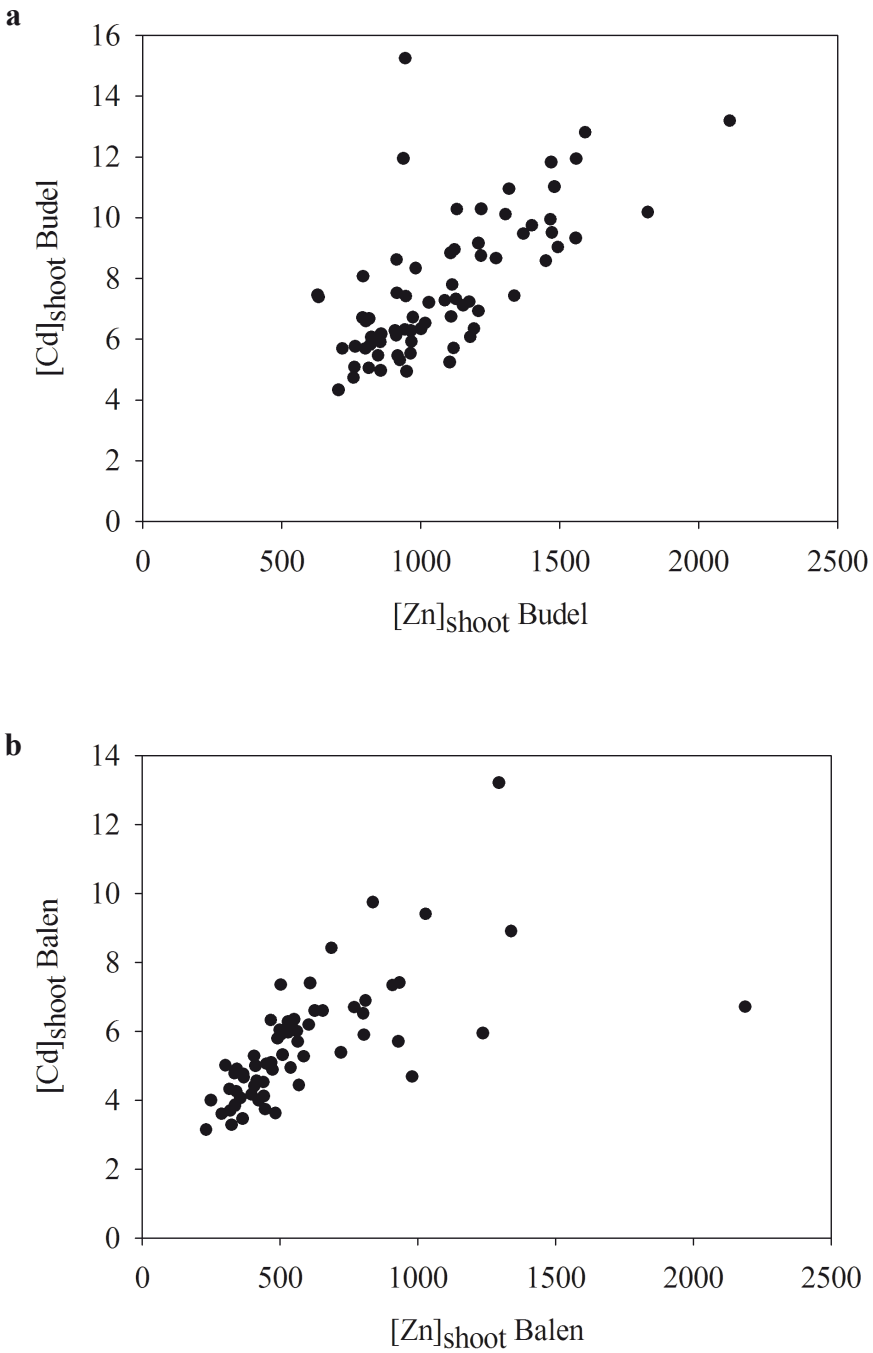

Figure 3. (a) Correlation of [Cd] shoot and [Zn] shoot (µg g-1 DW) in Budel (n=72). (b) Correlation of [Cd] shoot and [Zn] shoot (µg g-1 DW) in Balen (n=64).

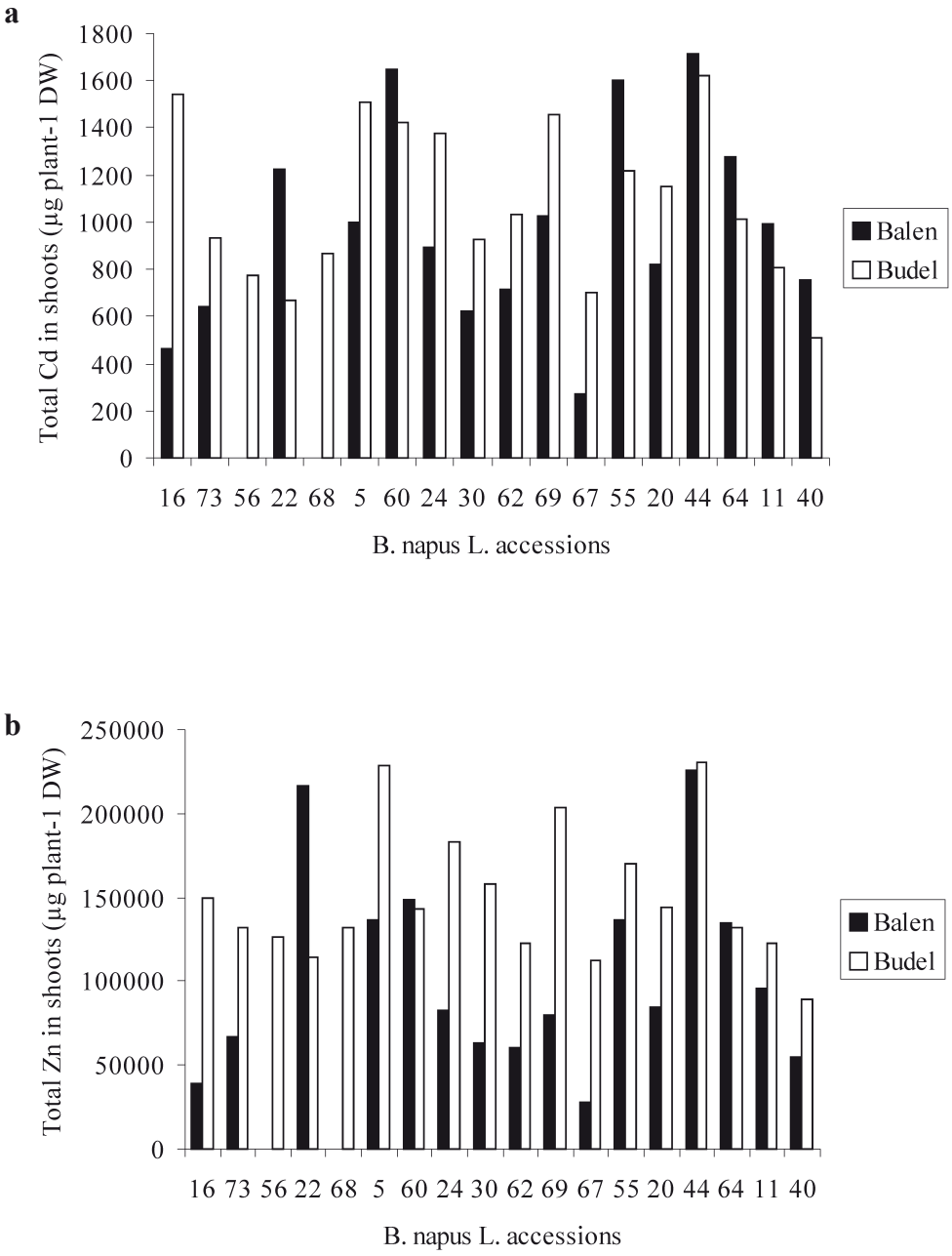

Figure 4. (a) Total Cd in shoots (µg plant-1 DW) of the selected B. napus L. accessions (n=4) at location Balen and Budel. (b) Total Zn in shoots (µg plant-1 DW) of the selected B. napus L. accessions (n=4) at location Balen and Budel.

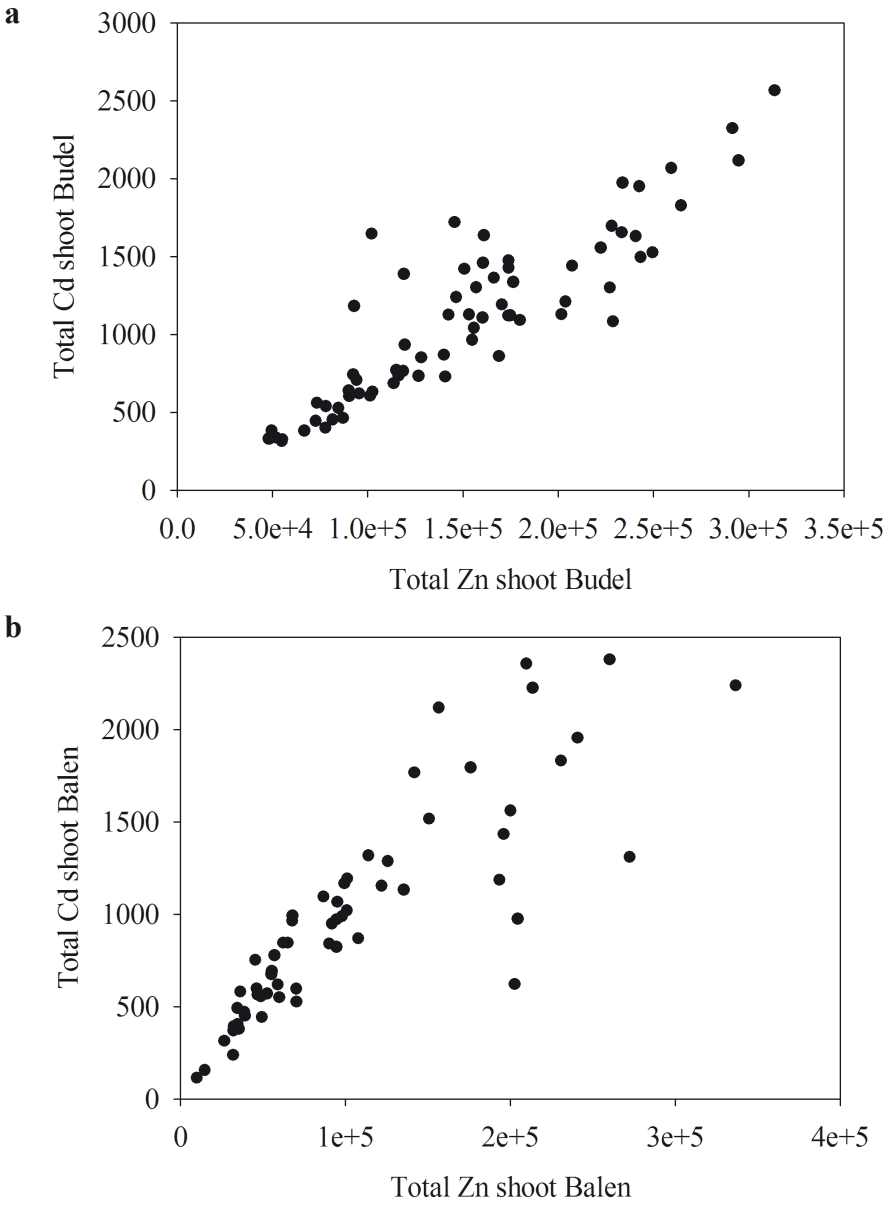

Figure 5. (a) Correlation of Total Cd in shoots and total Zn in shoots (µg plant-1 DW) in Budel (n=72). (b) Correlation of Total Cd in shoots and total Zn in shoots (µg plant-1 DW) in Balen (n=64).

(44 and 60) performed well at both locations regarding Total Cd and total Zn (Fig. 4a,b).

Correlation studies between the greenhouse and field experiments

When comparing the shoot Cd concentrations for the 18 *B. napus* accessions, we found no correlation between the greenhouse- and field results from Balen (Pearson correlation coefficient of -0.168 and a two-tailed significance of 0.534).

Discussion

Greenhouse experiment

Metal accumulation in shoots on hydroponics

Brassica species are well known as metal accumulators and especially *B. juncea* (Indian Mustard) has been investigated for several years for the accumulation of a range of metals in its shoots (Salt et al., 1995b; Huang and Cunningham, 1996; Salt and Raskin, 1996; Ebbs and Kochian, 1997; Salt et al., 1997; Ebbs and Kochian, 1998; Bennett et al., 2003; Jiang et al., 2004; Podar et al., 2004; Qadir et al., 2004; Wangeline et al., 2004; Banuelos et al., 2005; Belimov et al., 2005; Ghosh and Singh, 2005). In a preliminary screening the Cd accumulation traits were compared of three *Brassica* species i.e. *B. juncea*, *B. carinata* and *B. napus*. Based on this study, it was decided to proceed with *B. napus* accessions mainly because this species is an oil crop and the other two are not, but also because the variation in Total Cd, [Cd]shoot and R/S ratio were not significantly different among *B. juncea* and *B. carinata* (data not shown). Another important aspect is the close relatedness with Arabidopsis thaliana (Lagercrantz, 1998; Paterson et al., 2001) and Thlaspi caerulescens, known for its Zn and Cd hyper accumulating traits (Lombi et al., 2001; Zhao et al., 2002; Assuncao et al., 2003; Roosens et al., 2003). Furthermore *B. napus* is world wide used as an oil producing crop, which makes it a suitable crop to adopt

in existing farming systems and has the potential to turn phytoextraction into a profitable enterprise. The variation we found within the 77 accessions with respect to Total Cd, [Cd]shoot and S/R ratio was reasonable and sufficient to make an initial selection for suitable accessions for the extraction experiment in the field. The relatively low variation could be explained by the fact that *B. napus* is a crop species selected for many years for commercial characteristics (high seed production, oil content), leading to erosion of natural variation. This variation within *B. napus* is low compared to different populations of T. cearulescens (Zhao et al., 2003). Accessions with a high [Cd]shoot did not exhibit high Total Cd, confirming that biomass production plays a crucial role in effective phytoextraction (Klang-Westin and Eriksson, 2003). Also high [Cd]shoot did not consistently combine with a high S/R ratio. Therefore it was decided to select the accessions for the field experiment based on their Total Cd and S/R ratio.

Field experiment

Soil analysis

The selected sites in Balen en Budel are heterogeneous in total soil Cd and Zn concentrations. In contrast to Cd, the Zn water extractable fractions differ between the two sites, which could be due to the higher pH value (5.6 versus 4.2) and the higher Ca concentration in the Balen soil in comparison with the Budel soil. In similar studies of contaminated soils in the vicinity of Zn smelters in NW Switzerland and NE France, the total soil Cd and Zn levels are remarkably higher or similar to those found in Budel and Balen (Keller et al., 2003; Schwartz et al., 2003).

Phytoextraction of metals in the field

In contrast to strictly controlled circumstances in the greenhouse, soil characteristics such as Fe-, Al- and Mn-oxides, organic matter and pH play

a fundamental role in metal bioavailability in a field situation (Basta et al., 2005). For corn it was found that soil pH interacted significantly with sludge borne soil Cd contents to affect corn leaf Cd concentrations. Furthermore Cd uptake in radish increased with decreasing pH (John et al., 1972; Chaney et al., 1976). Two hyperaccumulator species, *Alyssum murale* and *A. corsicum*, exhibited increased Ni concentrations in shoots as soil pH increased despite a decrease in water-soluble soil Ni, opposite to what is seen in agricultural crops (Kukier et al., 2004). Additionally soil Pb concentrations exceeded the soil Cd concentrations in Balen, indicating a potential antagonistic role for Pb in the case of Cd plant uptake (Das et al., 1997) at this site. Water extractable soil Zn concentrations were higher in Budel compared to Balen, and accordingly this difference is also found in the average total amount of Zn in the shoots. Two accessions performed equally well on both locations regarding Total Cd and Zn uptake, suggesting potential candidates for phytoremediation purposes.

Correlation/competition between Cd and Zn

In low Zn polluted soil the plant Cd and Zn uptake are linked to each other, as shown by the significant correlations. Due to chemical and physical similarity both metals are likely to be taken up and/or stored via similar transporters (Lombi et al., 2001; Zhao et al., 2002). Pronounced interactions between Zn and Cd also occurred in Cd uptake and translocation in soybean (Chaney et al., 1976). Due to the relatively low total soil Zn concentration, competition between Cd and Zn did not occur, whereas high Zn diminishes Cd uptake e.g. in Thlaspi cearulescens Prayon (Roosens et al., 2003). Other studies have shown that in sites highly polluted with Cd, high soil Zn concentrations did not significantly lower Cd concentrations in lettuce and spinach. In this study it was also postulated that this is dependent on how homogenously or heterogeneously contaminated your growth substrate is. Heterogeneity of soil Zn contamination would favor Zn over Cd uptake (Podar et al., 2004).

Correlation between hydroponics study and field experiment

No apparent correlation was found between the results of the hydroponics experiment and the field experiment. In order to select suitable *Brassica* accessions for Cd accumulation, one could choose to select accessions under standard and therefore repeatable conditions using hydroponics (greenhouse) or to expose the accessions outside in the field, where the abiotic conditions are variable, but more realistic. The selection procedure as outlined in this manuscript using the hydroponics system might perhaps not be suitable. An important aspect could be the absence of root-soil interaction and respective rhizosphere mechanisms, as was suggested for Cd accumulation in grains of different wheat varieties under hydroponics and in the field (Greger and Lofstedt, 2004). Even more striking is the result of a QTL (Quantitative Trait Loci) analysis of RIL's (Recombinant Inbred Lines) of Arabidopsis thaliana, emphasising the different effects of a soil or hydroponics treatment. The plants were cultivated under similar environmental conditions (greenhouse) except for the growth substrate (soil versus hydroponics). In this study completely different QTL's with respect to metal uptake were expressed (pers. com. A. Ghandilyan). The results of our experiments are in line with these findings.

Future studies

To complement this study on the Cd extraction potential of *B. napus* a crossing experiment is being conducted to select for the traits high Total Cd and a beneficial shoot/root Cd concentration ratio. It is evident that selection for high Total Cd and high S/R ratio among the *B. napus* accessions is limited and will not lead to very promising *B. napus* lines. Therefore we decided to make crosses between the most promising lines and already obtained F2 families, which will be tested on hydroponics and in the field. Furthermore a few accessions are being transformed with candidate genes involved in uptake, accumulation and translocation of metals.

Acknowledgements

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Supplementary table 1: Overview of tested *B. napus* accessions, supplied by the Centre for Genetic Resources, The Netherlands and the USDA, ARS, NCPRIS, Iowa State University, United states			
CGN nr.	Name	Country of origin	Year
CGN 17373	Norli	Germany	1981
CGN 17372	Niederarnbacher	Germany	1953
CGN 06870	Slapska	Czech Republic	1974
CGN 17371	Mirander	Germany	1982
CGN 18957	Bristol	France	1992
CGN 18966	Diamant	Germany	1954
CGN 17362	Lirastern	Germany	1979
CGN18948	Andol	France	
CGN 17355	Lingot	France	1962
CGN 17365	Madora	Germany	1990
CGN 13914	Cascade	USA	1985
CGN 6871	Mestnij	Russia	1974
CGN 17357	Liquanta	Germany	1987
CGN 17358	Lirabon	Germany	1985
CGN 18952	Ariana	Germany	
CGN 6821	Dolnoslayski	Poland	1974
CGN 18955	Bienvenu	France	1988
CGN 13912	Expander	Germany	1976
CGN 18950	Arabella	Germany	1987
CGN 18956	Brilland	Poland	
CGN 18951	Argus	Sweden	
CGN 11013	Primor	France	1973
CGN 17363	Lirektor	Germany	1987
CGN 17385	Ceres	Germany	1984
CGN 6878	Vinnickij Mestnyj	Ukraine	1980
CGN 18964	Darmor	France	1984
CGN 17359	Lirajet	Germany	1990
CGN 18965	Diadem	Germany	1988
CGN 17374	Olimpiade	Italy	
CGN 17368	Marens	France	
CGN 17325	Fiona	Germany	
CGN 18954	Belinda	The Netherlands	1984
CGN 17381	Prominj	Russia	
CGN 6877	Dublianskij	Russia	1980
CGN 17380	Planet	Germany	
CGN 17333	Herkules	Sweden	
CGN 17364	Liropa	Germany	1983
CGN 17323	Falcon	Germany	1989
CGN 6868	Baltia	Russia	1974
CGN 17337	Jupiter	Sweden	
CGN 6881	Vinnickij 15/59	Ukraine	1980
CGN 11012	Mansholts Hamburger	The Netherlands	1899

CGN 17382	Ramses	France	1980
CGN 17370	Matador	Sweden	
CGN 17336	Janetzkis	Austria	
CGN 17369	Marex	Germany	
CGN 17354	Lindora	Germany	1983
CGN 17338	Kurander	Germany	1983
CGN 6880	Mytnickij	Ukraine	1980
CGN 6874	Niemierczansky	Russia	1974
CGN 17340	Ledos	Germany	1978
CGN 6879	Vinnickij 21	Ukraine	1980
CGN 17367	Maras	Poland	
CGN 17353	Ligora	Germany	1979
CGN 18961	Collo	Germany	1989
CGN 17384	Accord	Germany	1990
CGN 6869	Kromerska	Czech Republic	1974
CGN 17315	SKR. II Kormovoi	Russia	
CGN 17379	Perle	Germany	
CGN 17324	Fertodi	Hungary	
CGN 17308	Kombainer	Russia	
CGN 17377	Panter	Sweden	1968
CGN 17313	Kievskii 18	Russia	
CGN 17332	Heimer	Sweden	
CGN 17305	Shen-Li Jutsaj	China	
CGN 17316	Uspekh	Russia	
CGN 17329	Gungula	Germany	1983
CGN 17317	Blagodatnyi	Russia	
CGN 17375	Olymp	Germany	1990
CGN 18959	Capricorn	Germany	
CGN 17326	Gesunder	Germany	1971
CGN 18960	Cobra	Germany	1986
CGN 17327	Girita	Germany	1976
CGN 17318	Fedorovskii	Russia	
CGN 17314	Kombi	Russia	

USDA nr	*Botanical name*	*Item accession*	*Plant name*	*Country*
43 PI	Brassica napus	391553	Shang-You	China, Shaanxi
56 PI	Brassica napus	443015	Gry	Norway

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Chapter 3

A Crossing Experiment with *Brassica napus* L.: Searching for Enhanced Cadmium Accumulation

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Abstract

To obtain *Brassica napus* accessions with enhanced ability to accumulate cadmium (Cd) in the shoots we intercrossed accessions with the highest total shoot Cd content or with the the highest shoot to root Cd concentration ratios. These parent lines were selected on the basis of a hydroponic screening of 77 accessions. The obtained F2 crosses and the parent accessions were then tested on moderately contaminated soil originating from a field adjacent to a metal smelter in Belgium (Umicore Balen), in the greenhouse. Shoot Cd concentration ($[Cd]_{shoot}$) and shoot dry weight were quantified. One out of five F2 families tested exhibited significant two-sided transgression beyond the parent phenotypes, suggesting that there is some potential to improve shoot Cd accumulation through crossing and selection. Furthermore, shoot Cd content (μg $plant^{-1}$) was quantified and three out of five F2 families exhibited significant two-sided transgression beyond the parent phenotypes. This suggests that selection based on this parameter will more likely result in a higher biomass, rather than in a higher Cd concentration as such.

Introduction

Breeding and selection of plants has been going on for centuries, be it for enhancing yield, disease-resistance, or taste and color. For phytoremediation or food fortification purposes, there is currently a demand for plant species with increased capacity to transport heavy metals from their roots to the shoots. On the other hand, crops with decreased accumulation of non-nutritional toxic metals and metalloids in their edible parts would be desirable too, for the purpose of food quality improvement.

Successful breeding through crossing and selection requires the presence of heritable variation of the trait of interest. Intra-specific heritable variation of essential or non-essential metal or metalloid concentrations in seeds or leaves seems to be common in both crops and wild plants, including *Arabidopsis thaliana* (Graham et al., 1999; Vreugdenhil et al., 2004; Williams and Mills 2005; Williams and Mills 2005; Stolt et al., 2006; Tanhuanpaa et al., 2007; Kubo et al., 2008).

Brassica napus L. (*Brassicaceae*) has been suggested to have potential as a phytoremediator crop, because it combines high-biomass production and potential commercial value (rapeseed oil for biodiesel) with fair rates of metal accumulation in its above ground parts (Moroni et al., 2003; Purakayastha et al., 2008).

Earlier experiments, where 77 accessions of *B. napus* L., were tested for their Cd and Zn accumulation and translocation from root to shoot, in both hydroponics and field experiments, showed very little natural variation between the different accessions (chapter 2). For this reason it is important to find out whether there will be transgressive segregation of these properties in the progenies of inter-accession crosses, because selection from transgressing crosses would significantly speed up the selection procedure. More specifically, it is interesting to check whether transgression towards higher foliar Cd concentrations can be achieved through intercrossing *B. napus* accessions with high foliar Cd accumulation or high Cd translocation rates. Therefore, based on the results we obtained from the hydroponics and field experiment

(chapter 2), we intercrossed the highest Cd accumulating and the highest Cd translocating accessions, hoping to obtain F2 progeny with transgressively enhanced shoot Cd accumulation.

Material and Methods

Establishment of the crosses

Crosses were made between the 6 parent accessions (P), 3 of which showed the highest shoot Cd content, expressed as μg Cd plant^{-1} (P1, P2 & P4) and 3 that showed the highest shoot to root Cd concentration ratio (P9, P11& P14) in a hydroponics experiment with exposure for ten days to 0.2 μM $CdSO_4$. We made reciprocal F1 crosses of the following parent accession combinations; P1xP2, P1xP9, P2xP11, P4xP14 and P9xP11. F2 crosses were established through selfing. Subsequently they were germinated and grown on homogenized moderately contaminated soil from "Balen", Belgium. This site, located in the vicinity of a metal smelter (Umicore, Balen, Belgium), has been used in our previous field study (chapter 2) and the main pollutants in this soil are Cd and Zn.

Greenhouse experiment

Six winter oilseed rape accessions of *B. napus* L. and their F2 crosses were grown under standard conditions in the greenhouse (22/16°C day/night; 175 μmoles m^{-2} s^{-1} at plant level; 16h d^{-1}). Fourteen days after germination on homogenized Balen soil, the seedlings were transferred to single pots filled with the same soil. Each accession and each cross was represented by 11-19 and 33-41 plants, respectively, with one seedling per 2-L polyethylene pot. The seedlings were grown for another 14 days before harvest. At harvest shoots and roots were separated and dried for 48 h at 70°C in a stove, and weighed.

Plant analysis

Plant samples were wet-ashed in 2 ml of 37% HCl/65% HNO_3 (1/4 v/v) in Teflon bombs at 140°C for 7 hours. Shoot Cd concentration ($[Cd]_{shoot}$) was determined using graphite furnace AAS (Perkin Elmer 4100 ZL) after which the shoot Cd content (μg $plant^{-1}$) was calculated.

Statistical analysis

Statistic analysis was performed using two-way ANOVA. The MSR statistic was used for a posteriori comparisons of individual means (Rohlf and Sokal 1981). When necessary, data were subjected to logarithmic transformation prior to analysis. After testing for normality (significant skewness or other deviations from the normal distribution did not occur), the expected frequencies in the extreme 2.5-% area tails of the distribution (two-sided) (0.05 x n) were calculated for the parent lines. The expected frequencies of extreme phenotypes in the crosses, under the H0 hypothesis (= no transgression), were calculated in the most conservative way, i.e. as 0.75 x 0.05 x n (assuming 1 gene with full dominance in either direction), using the extreme 2.5-% area borders of the corresponding parent lines. The frequencies of individuals with extreme phenotypes were treated as Poisson-distributed variables. Observed frequencies were compared with expected ones and deviations were considered to be significant if the relative Poisson area was less then 5% of the area of the Poisson distribution with the expected number as the mean. To prevent any artifactual H0 rejection due to overall positive or negative effects of crossing, the analyses were repeated after shifting the F2 distribution in question along the abscissa until the mean was exactly intermediate between the parent means. This appeared to remove any significant one-sided transgression.

Results

Significant differences among parent lines

The parent accessions showed significant differences (Table 1) in their shoot Cd concentration (Table 2).

Table 1
Analyses of variance among and within parents for shoot Cd concentrations.

Source	df	SS	MS	F	p
between	5	85.799	17.160	3.92	< 0.01
within	85	371.709	4.373		

Table 2
Shoot Cd concentrations of the six parent accessions and five F2 crosses. Concentrations are expressed as µg metal g^{-1} shoot dry weight. Values represent averages ± SD, minimum and maximum values, n = number of plants analyzed.

Parent/Cross	Shoot Cd concentration (µg g^{-1} dw)	Min/max	N
P1	7.35 ± 0.83	6.10/8.90	11
P2	9.29 ± 2.25	6.94/14.97	11
P4	10.40 ± 2.13	6.55/13.60	15
P9	7.73 ± 2.09	4.79/12.84	19
P11	8.92 ± 2.02	5.93/12.83	14
P14	8.58 ± 2.43	6.11/15.09	21
P1xP2	9.18 ± 2.75	4.32/15.63	41
P1xP9	7.91 ± 2.56	4.16/14.95	41
P2xP11	7.79 ± 3.03	2.54/14.16	34
P4xP14	9.32 ± 2.12	4.93/13.37	39
P9xP11	8.33 ± 2.78	4.36/17.15	40

F2 crosses show significant transgression

Except for P4xP14, the standard deviations of the foliar Cd concentration distributions of the crosses were higher, though only slightly, than in the parent lines, which suggests a limited degree of segregation in most of the crosses (Table 2). There was only one significant case of transgression, P2 x P11, which was also the cross with the highest standard deviation (Table 2). In this cross 4 individuals were in the higher < 2.5-% area and 3 in the lower < 2.5-% area, both before and after shifting the distribution until the mean was intermediate between the parent means (Table 4). This case of transgression was highly significant ($P<0.001$), even after correction for multiple comparisons ($P<0.005$), whereas in the other crosses the frequencies of extreme phenotypes were very close to expectations. However, the analysis was performed most conservatively and therefore the overall degree of transgression could have been underestimated (Table 4). For shoot Cd content (Table 3), significant

transgression was found for all crosses, except one (Table 5).
The segregation for shoot biomass showed roughly the same pattern. There

Table 3
Shoot Cd content of the six parent accessions and five F2 crosses. Shoot Cd content is expressed as µg per plant. Values represent averages ± SD, minimum and maximum values, n = number of plants analyzed.

Parent/Cross	Shoot Cd content (µg per plant)	Min/max	N
P1	66.34 ± 13.21	55.49/78.26	11
P2	65.94 ± 13.57	55.07/108.70	11
P4	81.54 ± 14.69	51.36/106.76	15
P9	64.74 ± 13.38	44.64/114.30	19
P11	72.77 ± 12.70	52.70/98.78	14
P14	73.19 ± 14.28	54.00/100.05	21
P1xP2	70.05 ± 13.28	38.83/112.69	41
P1xP9	67.44 ± 15.70	32.39/96.75	41
P2xP11	65.31 ± 18.88	16.55/107.66	34
P4xP14	79.47 ± 18.04	37.20/124.42	39
P9xP11	65.85 ± 19.92	31.75/114.75	40

was significant transgression in all the crosses, with 4 ($P<0.05$), 10 ($P<0.001$), 4 ($P<0.05$) and 6 ($P<0.01$) individuals in the 5-% area tails for P1xP2, P1xP9, P2xP11 and P9xP11, respectively. Again, there was no significant transgression in P4xP14, with zero individuals in the 5-% area tails.

Discussion

For shoot Cd concentration, there was significant transgression in only one out of five F2 families, of which at least four seemed to segregate for this trait. This is rather low, even when considering that the degree of transgression might have been underestimated due to conservative testing. In general, in the absence of directional selection with contrasting directions in both parent accessions and a quantitative, polygenic inheritance of the trait in question, one would expect that both parent accessions would randomly contribute trait enhancing alleles at different loci, which would in turn lead to a high incidence of transgression. There are different potential reasons for the apparent absence of a high frequency of transgression of the shoot Cd concentration

Table 4
Analyses of transgression for shoot Cd concentrations.

Parent/cross	Expected in 5-% area (two-sided)	Observed in 5-% area (two-sided)	p
P1 + P2	1.1	1	0.36
P1 + P9	1.5	1	0.32
P2 + P11	1.3	1	0.35
P4 + P14	1.8	1	0.30
P9 + P11	1.7	0	0.17
P1 x P2	1.5	3	0.13
P1 x P9	1.5	2	0.25
P2 x P11	1.3	7	<0.001
P4 x P14	1.5	0	0.25
P9 x P11	1.5	2	0.25

Table 5
Analyses of transgression for shoot Cd content. *) this family was not normally distributed.

Parent/cross	Expected in 5-% area (two-sided)	Observed in 5-% area (two-sided)	p
P1 + P2	1.1	0	0.36
P1 + P9	1.5	1	0.32
P2 + P11	1.3	2	0.23
P4 + P14	1.8	0	0.17
P9 + P11	1.7	1	0.31
P1 x P2	1.5	14*	<0.001*
P1 x P9	1.5	4	<0.05
P2 x P11	1.3	5	<0.01
P4 x P14	1.5	2	0.25
P9 x P11	1.5	6	<0.01

character in this study. First, the segregation might be largely controlled by one single gene. Second, dependent on the number of genes involved and their interactions, the frequency of transgressing phenotypes might have been too low to yield significant deviations from expectations, based on the numbers of F2 phenotyped in this study. Third, the overall heritability of the character might have been low, as suggested by the relatively small difference between the standard deviations of the parent accessions and the F2 families. To check this possibility, we estimated the heritability of the variation among the parent accessions, using the variance partitioning method according to Falconer (Falconer 1981). This yielded a heritability estimate (h^2) of 0.17, which is far from significant. In view of the expected proportionality between the heritability and the response to selection, as well as the low incidence of transgression, these results suggest that classical selection and breeding for shoot Cd concentration is unlikely to produce substantial results within a limited number of generations, possibly except when starting from transgressively segregating populations. In the latter case, to identify the genotypes combining the trait-enhancing alleles of both parents with reasonable certainty, molecular markers, based on a QTL analysis, would be indispensable. However, in view of the low heritability, such an analysis would probably require extremely big segregating populations in order to be successful, although, on the other hand, the heritability for transgressively segregating F2 might be higher than for the variation among the parent accessions.

The results obtained with the shoot Cd contents, on the other hand, showed transgressive segregation in 4 out of 5 cases, or at least 3 out of 4 when discarding the family with non-normal distribution. The segregation patterns obtained for shoot Cd content were overall in conformity with those for shoot dry weight, which strongly suggests that shoot dry weight was the major determinant of shoot Cd content in all except one of the transgressing F2 families. This was supported by the absence of any significant correlation between the shoot Cd concentration and shoot dry weight (data not given). Thus, considering the low incidence of transgressive segregation for shoot Cd concentration itself, it seems that selection for enhanced shoot Cd content would generally lead

to higher biomass, rather than higher shoot Cd concentration. In addition, the heritability of the variation for the shoot Cd content trait appeared to be higher, though insignificantly, than that for shoot Cd concentration ($h^2 = 0.20$ and $h^2 = 0.17$, for shoot Cd content and shoot Cd concentration, respectively). The response to selection equals the product of the selection differential and the heritability. Since both Cd shoot dry weight and shoot Cd concentration exhibit low heritability, the selection differentials required to significantly improve the mean trait levels within few generations will be high. From the data obtained in this study however, it appears that even in the transgressing crosses there is only a limited degree of variation, which restricts the possibilities to apply high selection differentials. Overall, it seems that the possibilities to breed and select *B. napus* lines with significantly improved Cd phytoremediator properties are limited, and that genetic engineering strategies might be more successful on the short term (Chaney et al., 2007). However, to develop effective engineered phytoremediator crops, much more molecular and physiological knowledge the uptake, plant-internal transport and tolerance of metals is required. Thus far, the only example of effective phytoextraction by means of a transgenic crop has been achieved for mercury (Heaton et al., 2003; Meagher and Heaton, 2005). However, strategies for Cd, Zn and other metals still have to be developed, although there is currently a rapid progress in laboratory studies (Hanikenne et al., 2008).

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Chapter 4

Expression of the Arabidopsis Metallothionein 2b Enhances Arsenite Sensitivity and Root to Shoot Translocation in Tobacco

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Abstract

We expressed the *AtMT2b* gene under the 35 S cauliflower mosaic virus promoter in *Nicotiana tabacum* (Sr1), using leaf disc transformation. Arsenite tolerance and uptake, as well as arsenite-induced phytochelatin (PC) accumulation in roots were measured in transgenic lines, and compared to untransformed ('wild type') tobacco. Measured after 5 days of exposure, arsenite tolerance was slightly but significantly decreased in the transgenic lines compared to wild type. The highest *AtMT2b* expressing line exhibited a significantly decreased arsenic accumulation in roots, but an increased accumulation in shoots, while the total amount of arsenic taken up remained unchanged, suggesting that *AtMT2b* expression enhanced the arsenic root to shoot transport. The same transformant line also exhibited a decreased rate of phytochelatin accumulation in the roots, but the phytochelatin-SH to As molar ratio was higher than in wild type, suggesting that the lower arsenite tolerance in the transformant lines was not due to a potential shortage of cysteine for PC synthesis, imposed by expression of the transgene.

Introduction

Metallothioneins (MTs) are cysteine-rich, low-molecular weight, metal-binding proteins. In mammals MTs function in Zn homeostasis and protect against cadmium toxicity (Coyle et al., 2002). Higher plant metallothioneins are classified into four types, of which type 1, 2 and 3 are believed to function in Cu homeostasis and tolerance (Murphy and Taiz 1995; van Hoof et al., 2001; Guo et al., 2003; Roosens et al., 2004; Guo et al., 2008), and type 4 in Zn homeostasis (Lane et al., 1987; Guo et al., 2008). Nevertheless, all of them can bind a variety of metal and metalloid ions, including non-essential ones (Cobbett and Goldsbrough 2002). Therefore, plant MTs have been suggested to function in non-essential metal detoxification, next to essential metal homeostasis (Merrifield et al., 2004; Merrifield et al., 2006; Guo et al., 2008).

Algal metallothionein from *Fucus vesiculosus* has been shown to bind trivalent arsenic with high affinity *in vitro*, and was therefore considered to represent a putative model for *in vivo* arsenic binding and detoxification (Merrifield et al., 2004). Higher plant MTs have not been investigated for arsenite binding thus far, but it is likely that all of them will do so, at least *in vitro*, in view of the high affinity of arsenite for thiol groups. In most plants arsenite seems to be chiefly bound to phytochelatins (PCs), rather than MTs (Sneller et al., 1999; Raab et al., 2004; Raab et al., 2005). However, this does not exclude the possibility that MTs and PCs have overlapping functions in the detoxification of arsenic, such as suggested for Cu and Cd in *Arabidopsis thaliana* (Guo et al., 2008).

To answer the question of whether plant MTs are functioning in arsenic detoxification, we expressed *MT2b* cDNA from *A. thaliana*, *AtMT2b*, in tobacco, using the strong 35S cauliflower mosaic virus promotor. This particular MT gene was chosen because its homologue in *Silene vulgaris*, *SvMT2b*, seemed to be responsible for high-level Cu tolerance in copper mine populations of this species, albeit as a hypostatic determinant (van Hoof et al., 2001). We tested the effect of *AtMT2b* expression on arsenite tolerance, using

root growth as a toxicity end point, as well as on arsenite accumulation in root and shoot. In addition, in view of the possibility of repression of the PC synthetic pathway, imposed through enhanced cystein consumption in MT protein synthesis, we also measured PC accumulation in the roots.

Material & Methods

Vector construction, transformation and molecular characterization

All DNA recombinant techniques were performed according to the GATEWAY Cloning System. An EcoR1- Xho1 (forward primer: 5'-cgg aat tcc atg tct tgc tgt ggt gga agc tg-3' and reverse primer: 5'-ccg ctc gag cgg ctt cat ttg cag gta caa ggg ttg-3') fragment, corresponding to the *Arabidopsis thaliana* Col. metallothionein 2b coding sequence (TAIR Accession; gene 3355698), was cloned into pENTR4 (Invitrogen) through restriction with the corresponding enzymes and ligation with T4-ligase. Subsequently the *AtMT2b* gene was transferred from the pENTR4 into the destination vector pK2GW7 (Karimi et al., 2002) through LR reaction. This binary vector contained a *neomycine phosphotransferase II* (*nptII*) gene, which confers resistance to kanamycin in the transformed cells. The gene of interest is under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The binary vector was introduced into the strain C58 (pMP90) by electroporation.

Tobacco (*Nicotiana tabacum* Sr1.) seeds were obtained from the Plant Genetics Department, Free University of Amsterdam. Tobacco has been transformed using Agrobacterium mediated leaf disc transformation, the transgenic explants were regenerated on kanamycin containing medium (at a concentration of 300 mg/l). PCR was used to identify *AtMT2b* transgenic lines among the kanamycin-resistant lines obtained. The PCR primers used were the following: the forward primer was directed at the 35S promoter with the sequence 5'-cat tgc cca gct atc tgt cac-3', and the reverse primer was directed at the *AtMT2b* gene with the sequence 5'- ccg ctc gag cgg ctt cat ttg cag gta caa ggg ttg-3'. T0 plants were transferred to garden soil in the

greenhouse and transgene expression levels determined through quantitative RT-PCR on total leaf RNA, using the endogenous *Nicotiana tabacum* actine gene as a reference. The primers for *AtMT2b* were: 5'- gtg gct gtg gag gat gtg g-3' (forward) and 5'- tgt tgc ttt ctc agc aga tc-3' (reverse). Those for *Nt actin* were 5'-gct tga cac tgc caa gag cag-3' (forward), and 5'- agg act tct ggg cac cgg-3' (reverse).

Plant material and experimental design

Selfings of the transformant lines were established and T1 seeds collected. T1 seeds were germinated on garden soil; 3-week old seedlings were transferred to 1-L polyethylene pots (3 seedlings per pot) filled with half-strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 µM KCl, 25 µM H_3BO_3, 2 µM $ZnSO_4$, 2 µM Mn SO_4, 0.1 µM Cu SO_4, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe(Na)EDTA in demineralised water buffered with 2 mM MES, pH5.5, adjusted with KOH. Solutions were renewed weekly during pre-culture, under exposure to As (III) solutions were renewed twice a week. Before the start of the experiment all plants were checked for the *AtMT2b* transgene through DNA isolation and PCR. Plants were grown in a growth chamber (20/15°C day/night; light intensity, 240 µE m^{-2} s^{-1}, 14 h d^{-1}; R.H.: 75%). Arsenic was supplied as sodium arsenite, in a series of 0, 3, 6 and 12µM $NaAsO_2$.

For measurement of root elongation, the root system of 12 plants per treatment (1 plant per pot) of the two *AtMT2b* expressing lines (M9 and M10) and untransformed tobacco were stained with active coal powder and rinsed with demineralised water at the start of the exposure (Schat and Ten Bookum 1992). Five days after exposure root growth, i.e. the length of the longest unstained root segment, was determined.

Uptake and translocation of arsenite

Uptake and translocation of As (III) was determined by measuring As in root and shoot samples of 12 plants, pooled into 4 separate samples. Roots were rinsed with demineralised water and blotted dry with paper tissue. As was

extracted by digesting approximately 100 mg dried plant material in 2 ml of 37% (v/v) HCL: 65% (v/v) HNO_3 (1:4, v/v) in Teflon cylinders for 7 hours at 140 °C, after which the volume was adjusted to 10 ml with demineralised water. Arsenic concentrations were determined on a flame atomic spectrophotometer (Perkin-Elmer 2100; Perkin-Elmer Nederland, Nieuwerkerk a/d IJssel, the Netherlands), using a MHS-10 hydride system (Waters Nederland, Etten-Leur, the Netherlands).

Analysis of phytochelatins (PCs)

PCs were analyzed using root material of 12 replicas per plant line (wild type and M10), exposed to 12 μM As (III) for 5 days. The root material was processed immediately after harvesting. PC extraction was carried out according to Sneller and colleagues (Sneller et al., 2000) with slight modification (Wojas et al., 2008). In short, approximately 200 mg fresh material was homogenized in 1.78 ml of 6.3 mM ice – cold diethylene triamine pentaacetic acid (DTPA), 100 μl of 1 M NaOH and 100 μl of 6M Na BH_4 , using mortar, pestle and quartz sand. N-acetyl-L-cysteine (NAC) was added during homogenization as an internal standard to a final concentration of 10 μM. The homogenates were centrifuged for 5-15 minutes at 13000 rpm. Derivatisation was carried out according to Sneller and colleagues (Sneller et al., 2000). Four hundred and fifty μl of 200 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulphonic acid (HEPPS) buffer pH 8.2, containing 6.3 mM DTPA, was mixed with 10 μl of 20 mM monobromobimane. To this mixture, 250 μl of plant extract was added and derivatisation was carried out for 30 minutes in a water bath at 45 °C in darkness. The reaction was stopped by adding 300 μl of 1 M methanesulphonic acid. The sample was filtered through a Costar Spin-X centrifuge tube with a nylon filter (0.22 μm). HPLC analysis was performed as described in Sneller et al. 2000, where NPT were separated on a Nova-Pak C_{18} analytical column (6 nm, 4 μm, 3.9 x 300 mm, Waters catalogue no. 11695) at 37 °C, and eluted with a slightly concave gradient of methanol and water, both containing 0.1% trifluoroacetic acid,

with fluorescence detection. The injection volume was 20 µl; total time of analysis was 70 minutes.

Statistics

Statistic analysis was performed using two-way ANOVA. The MSR statistic was used for a posteriori comparisons of individual means (Sokal and Rohlf 1981). When necessary, data were subjected to logarithmic transformation prior to analysis.

Results

Production and characterization of transgenic *AtMT2b* plants

Thirteen kanamycin-resistant tobacco Sr1 lines were obtained after transformation with the *AtMT2b* construct. The transformant plant lines showed a PCR product when using primers against the 35S promoter and the *AtMT2b* gene (not shown). With the help of Q-PCR (Opticon) the different

Table 1
Relative expression of *AtMT2b* (fold difference with *NtAct*) in transformant tobacco lines.

Transformant line	*Fold difference with NtAct n=3*
M1	0.87
M2	0.04
M3	0.87
M4	0.62
M5	0.07
M6	1.00
M7	1.15
M8	no data
M9	0.41
M10	3.73
M11	1.41
M12	6.49
M13	0.76

AtMT2b expression levels were determined (Table 1). Transgenic T1 plants of the lines M9 and M10 were used for subsequent experiments. M10 expressed *AtMT2b* approximately 9-fold higher than M9.

AtMT2b plants show decreased tolerance to arsenite

Estimated from the root elongation response, M9 and M10 were significantly more sensitive to arsenite than wild type (Figure 1, Table 2.1). A posteriori testing (MSR) revealed that both *AtMT2b* transformant lines were significantly different from wild type only at 12 μM As (III) ($p < 0.001$).

Arsenite accumulation and translocation

Transformant line M10 accumulated significantly less ($p < 0.001$) As in the roots compared to wild type at 6 and 12 μM As (III). Line M9 was intermediate between M10 and Wt type, neither significantly different from Wt, nor M10

Table 2
Two-way analysis of variance of arsenite-imposed root growth inhibition (1), arsenic root accumulation (2), arsenic shoot accumulation (3) and shoot-root arsenic concentration ratio (4) in wild type (Wt) tobacco (*Nicotiana tabacum* Sr1) and the transformed tobacco plant lines, M9 and M10, after 5 days of arsenite exposure. Twelve plants are measured per plant line concerning root growth inhibition, the accumulation and translocation data are based on these twelve plants pooled into four separate samples and are log-transformed prior to statistical analyses.

1. Arsenite-imposed root growth inhibition

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.99	0.50	8.67	($p < 0.001$)
arsenite exposure	4	41.71	10.43	181.79	($p < 0.001$)
p-line x As (III)	8	1.41	0.18	3.08	($p < 0.005$)
within subgroups	165	9.47	0.06		

2. Arsenic root accumulation

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.06	0.03	6.02	($p < 0.001$)
arsenite exposure	2	1.29	0.64	132.67	($p < 0.001$)
p-line x As (III)	4	0.08	0.02	4.11	(NS)
within subgroups	27	0.13	0.01		

3. Arsenic shoot accumulation

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.23	0.12	8.68	($p < 0.001$)
arsenite exposure	2	0.96	0.48	35.71	($p < 0.001$)
p-line x As (III)	4	0.12	0.03	2.22	(NS)
within subgroups	27	0.36	0.01		

4. Shoot-root arsenic concentration ratio

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.330	0.165	11.766	($p < 0.001$)
arsenite exposure	2	0.006	0.003	0.230	(NS)
p-line x As (III)	4	0.170	0.042	3.022	(NS)
within subgroups	27	0.379	0.014		

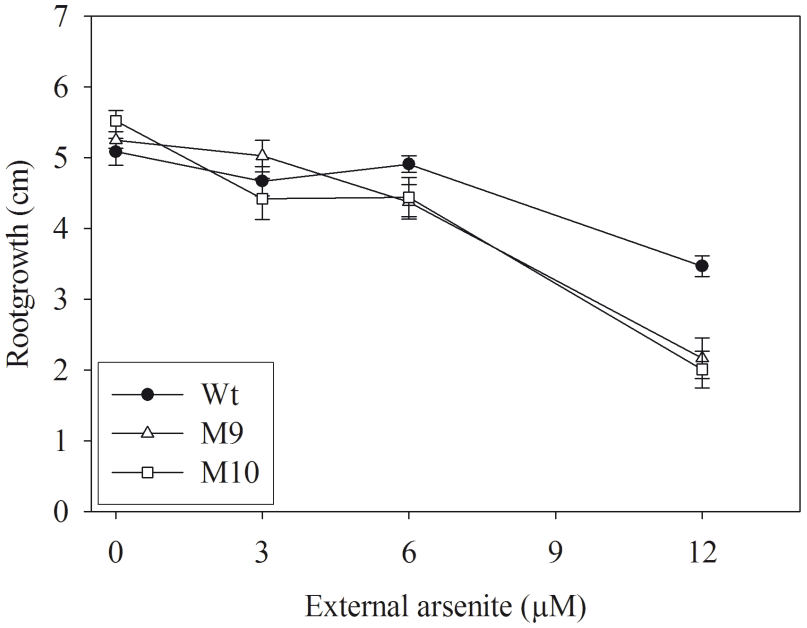

Figure 1. Arsenite imposed rootgrowth (cm) inhibition in wild type tobacco & AtMT2b expressing lines of transformed tobacco (mean ± S.E., n=12) exposed to 0-12 µM arsenite for 5 days.

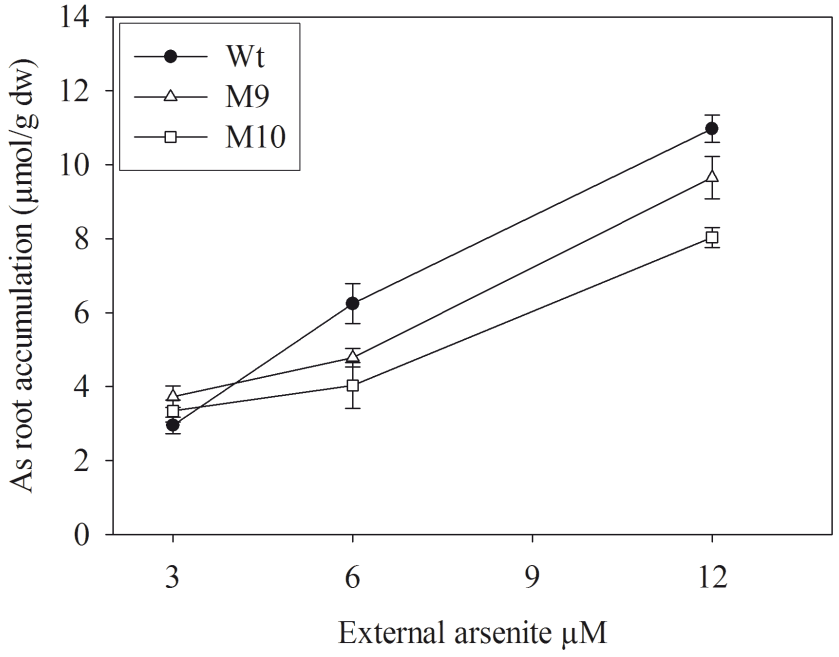

Figure 2. As accumulation in roots (µmol As/g dw) of wild type tobacco & At MT2b expressing lines of transformed tobacco (mean ± S.E., n=4) exposed to 3-12 µM arsenite for 5 days.

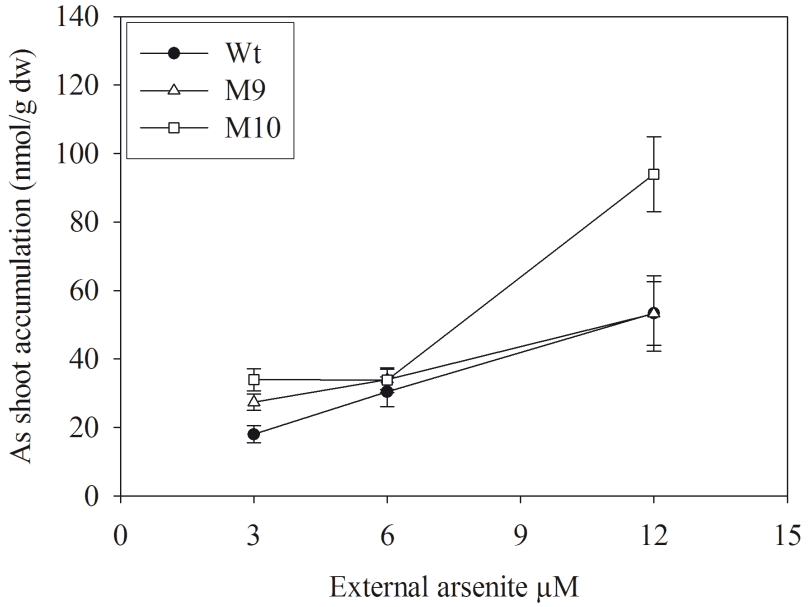

Figure 3. As accumulation in shoots (µmol As/g dw) of wild type tobacco & At MT2b expressing lines of transformed tobacco (mean ± S.E., n=4) exposed to 3-12 µM arsenite for 5 days.

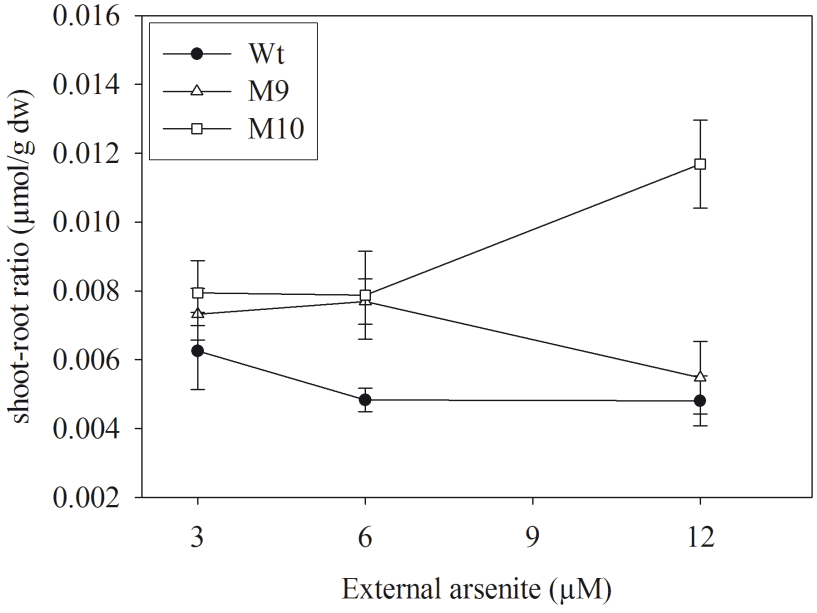

Figure 4. Shoot-root ratio (µmol As/g dw) of wild type tobacco & At MT2b expressing lines of transformed tobacco (mean ± S.E., n=4) exposed to 3-12 µM arsenite for 5 days.

(Figure 2, Table 2.2). As accumulation in the shoots was however significantly higher in M10 ($p < 0.001$) than in M9 or the wild type at 12 µM As(III) (Figure 3, Table 2.3). A significantly higher shoot to root As concentration ratio ($p < 0.001$) was also observed for M10 at 12 µM As (III) (Figure 4, Table 2.4). However, the total As burden per plant was insignificantly lower than in wild type at 6 and 12 µM As(III) (data not shown). Shoot and root dry weights and the root to shoot dry weight ratios were not significantly affected by As(III) exposure, and the corresponding treatment x plant type interactions were consistently insignificant, showing that the phenotypes for As accumulation in roots and shoots can not be attributed to differential effects of As(III) exposure on biomass productivity or allocation (data not shown).

Phytochelatin accumulation

In a separate experiment, PC's were analyzed in root material of wild type and M10, exposed to 12 µM As (III) for 5 days (12 replicas per plant line). The total PC concentrations were significantly higher in wild type. However, the molar PC-SH to As ratio in line M10 was significantly higher than in Wt (Table 3).

Discussion

Table 3
Glutathione, PC2 and PC3 concentrations (µmol g^{-1} root dw) and the PC-SH to As molar ratio, for roots of Wt tobacco (mean ± S.E., n=6) and the transformed line M10 (mean ± S.E., n=12) after 5 days of 12 µM As(III) exposure.

As (III) 12 µM	*Wt*	*M10*
GSH	4.93 ± 0.15	4.85 ± 0.38
PC2	8.31 ± 0.63	6.43 ± 0.35
PC3	9.25 ± 0.92	7.44 ± 0.71
PC-SH to As molar ratio	1.35 ± 0.14	1.70 ± 0.10

Our results clearly show that *AtMT2b* expression decreased arsenite tolerance, and enhanced the root to shoot arsenic translocation, without interfering with arsenite uptake into the roots, at least in the highest expressing transformant line. Admittedly, the arsenite sensitivity phenotype is moderate, but still contrary

to our expectation that MTs could contribute to As(III) sequestration.

Previous analyses have revealed that *MT2b* expression in *A. thaliana* is particularly high in phloem tissues, suggesting that the protein maybe primarily involved in the scavenging and redirection of Cu from senescing tissues (Guo et al., 2003). When expressed under the 35S CAM promotor, however, the gene will be expressed at high levels in other tissues too, including the rhizodermis and the root tip. In this way, the protein might interfere with the predominant pathways of arsenic detoxification in root cells. Arsenic detoxification depends strongly on PC synthesis, as shown by the strong As-hypersensitivity phenotypes of PC synthase-deficient Arabidopsis mutants (Ha et al., 1999), and of plants treated with buthionine sulfoximine (Hartley-Whitaker et al., 2001; Bleeker et al., 2006). In particular, As(III)-PC complex formation seems to be required for vacuolar arsenic sequestration in root cells (Bleeker et al., 2006). In this way, *MT2b* expression in the rhizodermal or root cortical cells could interfere with vacuolar sequestration through binding part of the arsenite, thus keeping it in the cytosolic compartment. This could in turn keep the arsenite available for radial transport across the root and subsequent loading into the xylem, which would explain the translocation phenotype of the highest *AtMT2b*-expressing transformant line. In any case, the sensitizing effect of *AtMT2b* expression does not seem to result from diverting cysteine away from the PC synthetic pathway. However, the GSH concentration was not decreased in M10, as compared to WT, which argues against a rate-limiting role for cysteine. The enhanced PC-SH to As molar ratio in M10 may be due to the lower As concentration in its roots. In general, PC-SH to metal molar ratio's tend to decrease with increasing metal accumulation rates (Schat et al., 2002). Alternatively, though less likely, the translocation phenotype could result from a disturbed redirection of arsenic from shoot to root via the phloem. Another possibility is that *AtMT2b* expression in the rhizodermis would interfere with arsenite efflux from the roots, which has recently been demonstrated to represent a major arsenic detoxification mechanism in rice and tomato (Xu et al., 2007). However, if this were the case, then one should expect an increased net rate of arsenite accumulation in the transformant.

However, we found an insignificantly decreased rate instead, which makes it unlikely that the transgene effect was due to interference with arsenite efflux.

Conclusion

In contrary to our expectation, *AtMT2b* expression decreased arsenite tolerance and enhanced arsenic root to shoot translocation in tobacco. More detailed research is needed to understand this effect.

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Chapter 5

Combined Expression of the *Arabidopsis* Metallothionein MT2b and the Heavy Metal Transporting ATPase HMA4 Enhances Cadmium Tolerance and the Root to Shoot Translocation of Cadmium and Zinc in Tobacco

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Abstract

We expressed the *AtMt2b* and *AtHMA4* genes under the 35 S cauliflower mosaic virus promoter simultaneously in *Nicotiana tabacum* (SR1), using leaf disc transformation. A single *AtMT2b* tobacco T2 line was used for re-transformation with *AtHMA4* to obtain the double transformant. Cadmium (Cd) and zinc (Zn) tolerance, uptake and translocation were measured in the double transformant, and compared to untransformed ('wild type') tobacco and single gene transformants. The double transformant exhibited enhanced Cd-tolerance, enhanced Cd and Zn root to shoot transport, but unaltered Zn tolerance and Cd and Zn uptake, compared with wild type.

The single transformant lines did not show significant phenotypes. Our results suggest that the phenotypes of the double transformant are due to synergistic interaction between the transgenes. Except for Cd tolerance, the phenotypes were moderate for Cd and Zn root to shoot transport, which may be due to use of the 35S promotor, resulting in incorrect tissue-specificity.

Introduction

The molecular mechanisms underlying heavy metal tolerance and heavy metal hyperaccumulation in plants have been increasingly studied over the past decade, partly because of the potential applicability of transgenic hyperaccumulator crops in the phytoremediation of metal-polluted soils (Verbruggen et al., 2009). The great potential of the genetic engineering approach in phytoremediation has been demonstrated for mercury (Hg) (Rugh 2004), although the asscociated enhanced Hg volatilization, due to expression of bacterial *merA* genes, make public acceptance difficult (Meagher and Heaton 2005). There is a huge array of literature describing the expression in plants of single genes with a supposed beneficial effect on metal tolerance or accumulation, i.e. mainly those involved in metal transport or chelation or in oxidative stress defence (Pilon-Smits and Pilon 2002). Usually, the phenotypes obtained are moderate or faint, if not absent or even directed towards lower tolerance or accumulation (Wojas et al., 2008). This may be owed to the complexity of metal tolerance and (hyper)accumulation traits, which probably depend on gene networks, rather then on single genes. Cross-species transcriptome comparisons between hyperaccumulating and related non-hyperaccumulating species consistently showed differential expression of a broad array of metal homeostatic genes (Becher et al., 2004; Hammond et al., 2006; Talke et al., 2006; van de Mortel et al., 2006), and QTL analysis of hyperaccumulator x non-hyperaccumulator crosses, or crosses between hyperaccumulator accessions with different accumulation capacities, consistently yielded multiple QTLs for metal accumulation or tolerance (Assuncao et al., 2006; Deniau et al., 2006; Courbot et al., 2007; Willems et al., 2007). Therefore, gene stacking through multiple transformation or tandem expression of different metal homeostatic genes may be more rewarding, as for example demonstrated for insect-resistance or herbicide-tolerance in maize and cotton (Halpin 2005). Among the genes that are consistently over-expressed in hyperaccumulators compared with non-accumulators are *HMA4* and several *MT* genes, among which MT2b (van de Mortel et al., 2006; Hanikenne et al.,

2008; Hassinen et al., 2009). *AtHMA4* encodes a stelar Zn and Cd transporting ATPase, which is involved in Zn xylem loading in *Arabidopsis thaliana* (Hussain et al., 2004). The strongly enhanced expression of the orthologous *AhHMA4* in the congeneric hyperaccumulator, *A. halleri*, has been shown to be essential for the Cd and Zn hypertolerance and foliar Zn hyperaccumulation traits in this species (Hanikenne et al., 2008). This gene also collocated with a major common QTL for Zn and Cd tolerance in a segregating backcross between *A. halleri* and the non-tolerant, non-hyperaccumulating congenitor, *A. lyrata* (Courbot et al., 2007; Willems et al., 2007). *HMA4* is certainly a key gene in the hyperaccumulator phenotype, but orthologous genes are also consistently over-expressed in Cd- and Zn-hypertolerant accessions of non-hyperaccumulator metallophytes, e.g. *SvHMA4* and *SpHMA4* in *Silene vulgaris* and *S. paradoxa*, in comparison with non-metallicolous accessions of these species (M. Arnetoli and H. Schat, unpublished). Likewise, *MT2b*-orthologous genes are over-expressed in hyperaccumulators, particularly in metallicolous accessions (Hassinen et al., 2009), in comparison with non-hyperaccumulators (van de Mortel et al., 2006), but also in metallicolous accessions of the non-accumulator metallophyte, *S. vulgaris*, in comparison with non-metallicolous accessions of this species (Mengoni et al., 2003; Jack et al., 2007). In the latter species it seems to function as a hypostatic metal tolerance 'enhancer', providing high-level tolerance in combination with some yet unknown epistatic gene or genes (van Hoof et al., 2001; Jack et al., 2007), although it doesn't seem to produce significant tolerance by itself (Hassinen et al., 2009). In a previous experiment it was even found that over-expression of AtMT2b in tobacco caused a slight, but significant sensitization to arsenite (Grispen et al., 2009). To test whether stacking of the two transgenes, *AtMT2b* and *AtHMA4*, would result in higher tolerance to or increased root-to-shoot translocation from root to shoot of Cd and Zn, we expressed *MT2b* and *HMA4* cDNA from *A. thaliana*, using the 35S cauliflower mosaic virus promoter, in tobacco, both alone and together.

Materials & Methods

Vector construction, transformation and molecular characterization of single AtHMA4 and double transformants of tobacco

All DNA recombinant techniques were performed according to the GATEWAY Cloning System. We obtained a cDNA clone, *AtHMA4*, from Riken, Japan (Resource number pda 08214, cDNA clone RAFL 09-32-D05). An amplicon suitable for TOPO cloning (forward primer: 5'-cac cat ggt cct aac act tct ctc aac ctt-3' and reverse primer: 5'-cgg cat tca cgg aat gag ac-3') was cloned in ENTR clone, pENTR D-TOPO. The construct was then sequenced using the Big Dye Terminator kit ABI on a 3700 ABI sequencing machine. Subsequently the *AtHMA4* gene was transferred from the pENTR D-TOPO into the destination vector pH2GW7 (Karimi et al., 2002) through LR reaction. This binary vector contained a hygromycin phosphotransferase (hpt) gene, which confers resistance to hygromycin in the transformed cells and our gene of interest is under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The binary vector was introduced into the *Agrobacterium tumefaciens* strain C58 (pMP90) by electroporation.

Tobacco (*Nicotiana tabacum* Sr1.) seeds were obtained from the Plant Genetics Department, Free University of Amsterdam. In the case of the *AtHMA4* gene, wild type tobacco has been transformed using Agrobacterium mediated leaf disc transformation, the transgenic explants were regenerated on hygromycin containing medium (at a concentration of 150 mg/l). PCR was used to identify *AtHMA4* transgenic lines among the hygromycin-resistant lines obtained. The PCR primers used were the following: the forward primer was directed against the 35S promoter with the sequence 5'-cat tgc cca gct atc tgt cac-3', and the reverse primer was directed at the *AtHMA4* gene with the sequence 5'-cgg cat tca cgg aat gag ac-3'. T0 plants were transferred to garden soil or agar in the greenhouse and the transgene expression levels determined through q-PCR on total RNA, using a *N. tabacum* actine as a reference gene (Grispen et al., 2009). The T2 35S::AtMT2b tobacco seeds were available from our own collection (transformant line M10; fold difference with *NtAct* is 3.73)

(Grispen et al., 2009); they were germinated and grown on garden soil and agar, and checked by PCR for the AtMT2b transgene. Subsequently leaves of these plants were used for re-transformation, by leaf disc transformation, with the *AtHMA4* gene under the control of the 35S promoter (see above).

Plant material and experimental design

Double transformants were self-pollinated by hand and T1 seed collected. Two separate experiments were done. In experiment 1 we exposed wild type tobacco (N*icotiana tabacum* Sr1), a single AtMt2b transformant (M10) and a double AtMT2b-AtHMA4 transformant (M+H) to Cd (0-0.1-0.4-1.6-6.4-25.6 μM) for 10 days. The group of plants that we exposed to Zn, were unfortunately not able to be used for further analyses because of an insect larvae attack, *Sciaridae* (*Diptera* family), feeding on the shoot just above the root, killing the plants (W.H.O. Ernst, personal communication). T1 seeds for M+H and T2 seeds for M10 were germinated on garden soil; 3-week old seedlings were transferred to 1-L polyethylene pots (3 seedlings per pot) filled with half-strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 μM KCl, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM Mn SO_4, 0.1 μM Cu SO_4, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA in demineralised water buffered with 2 mM MES, pH5.5, adjusted with KOH. Solutions were renewed weekly during pre-growth of two weeks; during exposure to $CdSO_4$, solutions were renewed twice a week.

In experiment 2 we exposed wild type tobacco, a single AtHMA4 transformant (H) and a double transformant (M+H) to Cd (0-0.4-1.6-6.4-25.6 μM) and Zn (0.05-32-64-128 μM) for 10 days. T1 seeds for (H) and (M+H) and T2 seeds for M10 were germinated on agar (the larvae that attacked our seedlings in experiment 1, probably hatched in the garden soil), containing kanamycin and hygromycin in case of the double transformant, hygromycin in case of the single transformant and no antibiotics in the case of wild type. The 3-week old seedlings were then transferred to hydroponics and treated the same as described in experiment 1.

Before the start of the experiment all plants were checked for the *AtMT2b* and

AtHMA4 transgene through DNA isolation and PCR. Plants were grown in a growth chamber (20/15°C day/night; light intensity, 200 $\mu E\ m^{-2}\ s^{-1}$, 14 h d^{-1}; R.H.: 75%). For measurement of root elongation, the root system of 12 plants per treatment (1 plant per pot) of the two transgene lines (single and double transformant) and wild type tobacco were stained with active coal powder and rinsed with demineralised water at the start of the exposure (Schat and Ten Bookum 1992). Ten days after metal exposure root growth, i.e. the length of the longest unstained root segment, was determined.

Uptake and translocation of Zn & Cd

Uptake and translocation of Zn & Cd was determined by measuring Zn and Cd in root and shoot samples of 12 plants, pooled into 4 separate samples. Before harvest the roots were desorbed with ice-cold 5-mM $PbNO_3$ for 1 hr. Roots were rinsed with demineralised water and blotted dry with paper tissue. One hundred mg of dried plant material was digested in 2 ml 37% (v/v) HCL: 65% (v/v) HNO_3 (1:4, v/v) in Teflon cylinders for 7 hours at 140 °C, after which the volume was adjusted to 10 ml with demineralised water. Zn and Cd concentrations were determined on a flame atomic spectrophotometer (Perkin Elmer 2100).

Statistics

Statistic analysis was performed using two-way ANOVA. The MSR statistic was used for a posteriori comparisons of individual means (Rohlf and Sokal 1981). When necessary, data were subjected to logarithmic transformation prior to analysis.

Results

Production and characterization of double transformants

Two hygromycin resistant tobacco Sr1 lines were obtained after transformation with the AtHMA4 construct (H1 & H2). The transformant plant lines showed a PCR product when PCR was conducted using primers against the 35S promoter

and the AtHMA4 gene. With the help of Q-PCR (Opticon) the different AtHMA4 expression levels were determined (Table 1). Three kanamycin and hygromycin resistant SR1 lines were obtained after re-transformation of the AtMT2b containing plant line (M10) with the AtHMA4 construct. The double transformant plant lines (M+H) showed a PCR product when PCR was conducted using primers against the 35S promoter, the AtMT2b gene and the AtHMA4 gene. Using Q-PCR (Opticon) the different AtMT2b and AtHMA4 expression levels were determined (Table 1). Transgenic T1 plants of line M+H1 and H2, and transgenic T2 plants of M10 were used for subsequent experiments.

Increased tolerance to Cd in the double transformant

Root growth inhibition was significantly lower in the double transformant compared to wild type and the two single transformants, M10 and H2, when exposed to Cd ($p<0,001$; figure 1 and 2(a) & table 2.1-2.2). When comparing the control root growth for Wt, M10 and M+H1 in experiment 1 and Wt, H2 and M+H1 in experiment 2, we found a two-fold difference, which could be due to the different germination conditions, garden soil versus agar. Under Zn

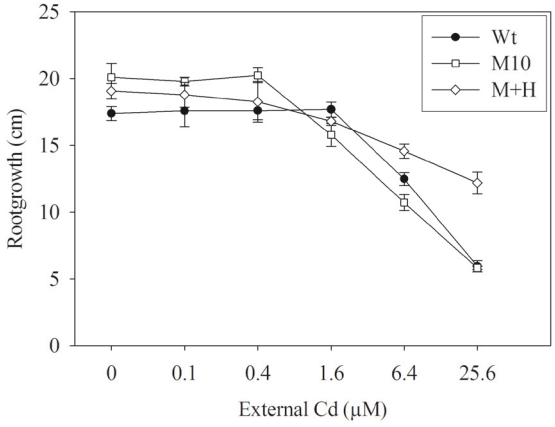

Figure 1. Cadmium imposed rootgrowth inhibition in Wt tobacco, AtMT2b (M10) and AtMT2b&AtHMA4 (M+H) expressing lines of transformed tobacco after 10 days of CdSO4 exposure.

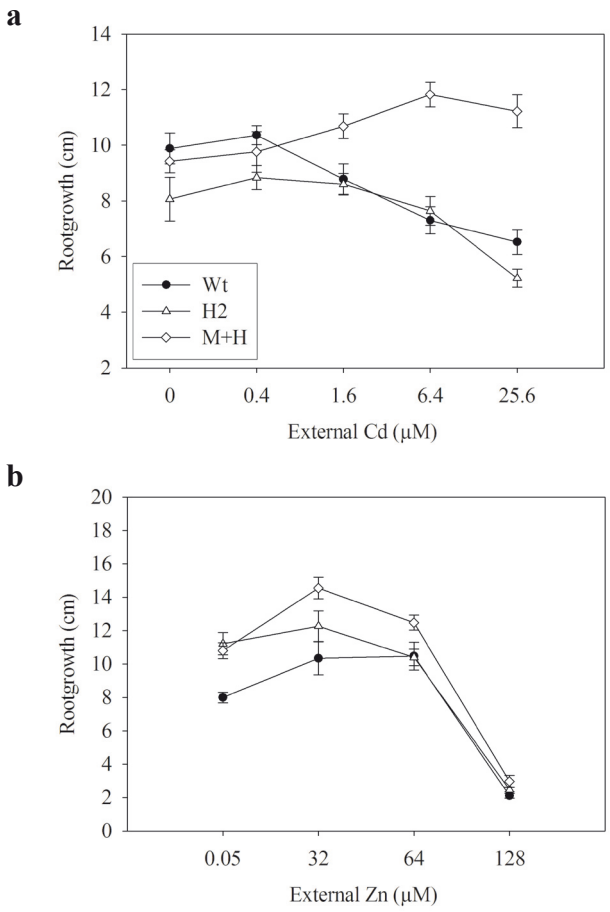

Figure 2. (a) Cadmium and (b) zinc imposed rootgrowth inhibition in Wt tobacco, AtHMA4 (H2) and AtMT2b&AtHMA4 (M+H) expressing lines of transformed tobacco after 10 days of CdSO4 exposure.

exposure, we found no significant difference between the double transformant and wild type or H2 (Figure 2(b)). For M10 we have no results concerning Zn exposure (see above). Cd and Zn-induced chlorosis and growth inhibition were consistently apparent at the highest exposure levels; however, there were no significant differences between the transformants, including the double one, and wild type (data not shown).

Increased translocation to the shoot of Cd and Zn in the double transformant

Translocation of Cd from the roots to the shoots was significantly higher in the double transformant compared with wild type and the two single transformants, M10 and H2 ($p<0,001$; figure 3 & 4(a) and table 2.3 &2.4). Translocation of Zn was also significantly higher in the double transformant compared to wild type and H2 ($p<0,001$; figure 4(b) and table 2.5), although

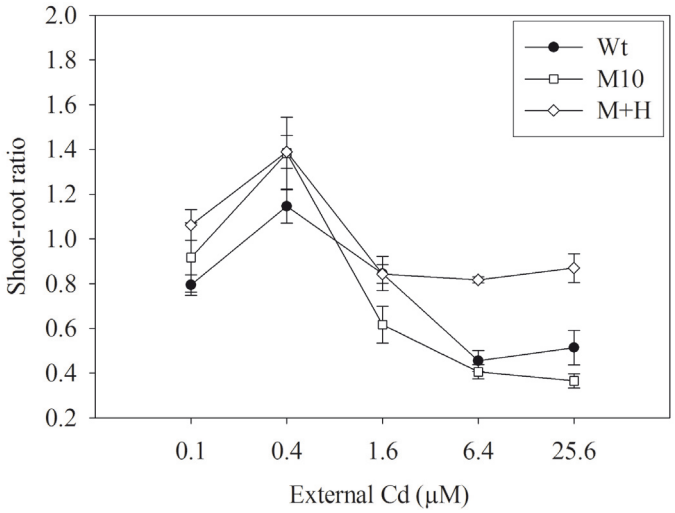

Figure 3. Shoot-root ratio (µmol Cd/g dw) for Wt tobacco, AtMT2b (M10) and AtMT2b&AtHMA4 (M+H) transformed tobacco.

only at the highest Zn concentration, 128 µM, as indicated by the significant Zn concentration x plant type interaction ($p<0,001$; figure 4(b) & table 2.5).

Shoot concentrations of Cd were significantly higher in the double transformant compared with wild type and the single transformant H2 ($p<0,01$; figure 5(a) & table 2.6), but not significantly different from the single transformant M10 (data not shown). The shoot Zn concentration was significantly higher in the double transformant compared to wild type ($p<0,05$; figure 5(b) & table 2.7), but not in comparison with the single transformant H2.

Root concentrations of Cd were significantly lower in the double transformant compared with wild type and the single transformant H2 ($p<0,01$; figure 6(a) & table 2.8), but not significantly higher than in the single transformant M10 (data not shown). Concerning Zn, the double transformant showed a significant

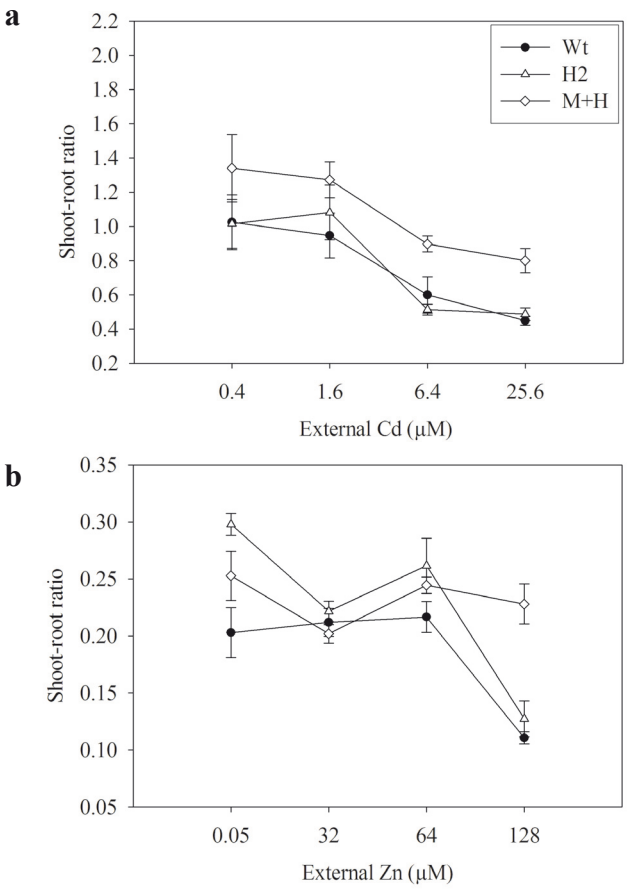

Figure 4. (a) Shoot-root ratio (µmol Cd/g dw) for Wt tobacco, AtHMA4 (H2) and AtMT2b&AtHMA4 (M+H) transformed tobacco. (b) Shoot-root ratio (µmol Zn/g dw) for Wt tobacco, AtHMA4 (H2) and AtMT2b&AtHMA4 (M+H) transformed tobacco.

lower root concentration compared with wild type and the single transformant line H2 ($p<0,001$; figure 6(b) & table 2.9), although only at the highest Zn concentration, 128 µM, as indicated by the significant Zn concentration x plant type interaction ($p<0,01$; figure 6(b) & table 2.9).

We found no phenotype for the total plant metal uptake (µmol/g plant dw) for either Cd or Zn exposure (data not shown).

Discussion

Our results show that the combined expression of *AtMT2b* and *AtHMA4*

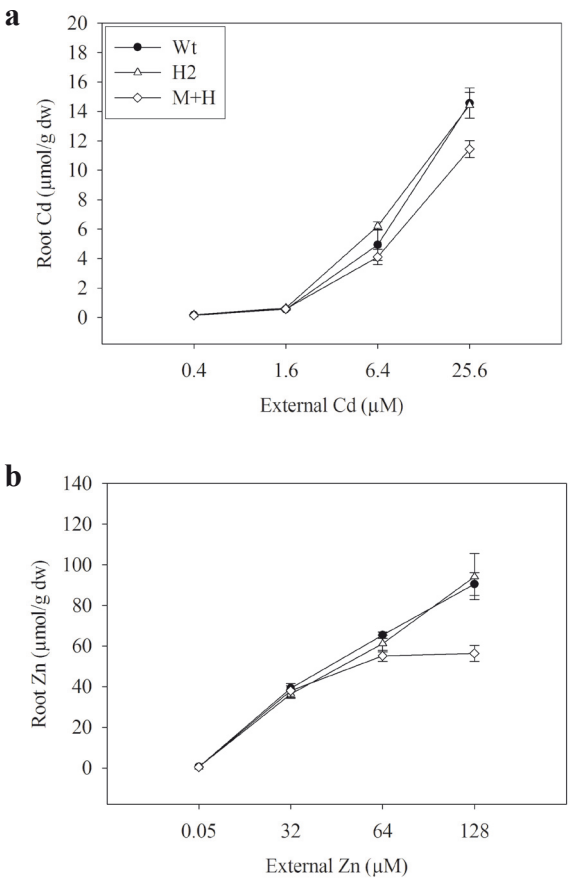

Figure 5. (a) Shoot Cd and (b) shoot Zn concentration (µmol Cd/g dw) for Wt tobacco, AtHMA4 (H2) and AtMT2b&AtHMA4 (M+H) transformed tobacco

significantly increased Cd tolerance, and significantly enhanced the root to shoot translocation of Cd and Zn, whereas no significant phenotypes were found in the single transformant lines. This might be attributed to a synergism between the transgenes. One alternative explanation, higher expression of either of the transgenes in the double transformant, does not seem to apply, because the expression levels in the single transformant lines were as high as, or even slightly higher than in the double transformant line (Table 1). However, we did not measure the protein concentrations, which, for whichever reason, might have differed between the single transformants and the double one. More extensive experiments, involving more lines and

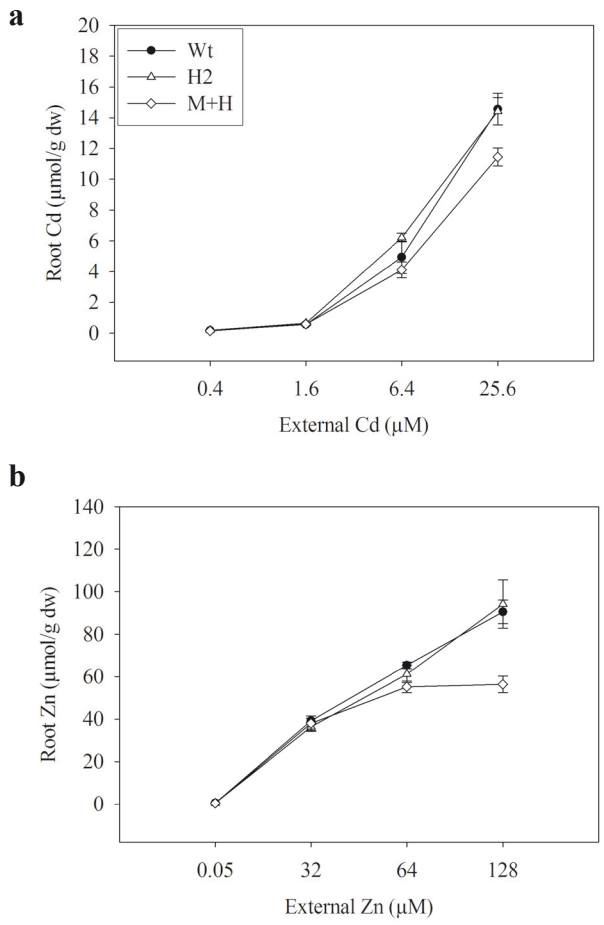

Figure 6. (a) Root Cd and (b) root Zn concentration (µmol Cd/g dw) for Wt tobacco, AtHMA4 (H2) and AtMT2b&AtHMA4 (M+H) transformed tobacco

protein quantitation should provide the hard evidence in favor or against the seemingly synergic interaction between the transgenes. Anyway, the present results are in agreement with the 'hypostatic enhancer' hypothesis for MT2b (van Hoof et al., 2001). According to this hypothesis, one would not expect a significant tolerance phenotype in lines exclusively expressing *MT2b*. In the present experiment we used root elongation as an end point for tolerance, which is justified in non-hyperaccumulators. However, root Cd accumulation was significantly lower in the double transformant than in wild type and in the single transformants, due to enhanced metal translocation to the shoot,

Table 2

Two-way analysis of variance for the four plant lines, wild type (Wt) tobacco (*Nicotiana tabacum* Sr1) and the transformed tobacco plant lines, M10, H2 and M+H. Cadmium-imposed root growth inhibition for Wt, M10 and M+H (1), cadmium-imposed root growth inhibition for Wt, H2 and M+H (2), shoot-root cadmium concentration ratio for Wt, M10 and M+H (3), shoot-root cadmium concentration ratio for Wt, H2 and M+H (4), shoot-root zinc concentration ratio for Wt, H2 and M+H (5), shoot cadmium concentration for Wt, H2 and M+H (6), shoot zinc concentration for Wt, H2 and M+H (7), root cadmium concentration for Wt, H2 and M+H (8) and root zinc concentration for Wt, H2 and M+H (9), after 10 days of either cadmium or zinc exposure. Twelve plants are measured per plant line concerning root growth inhibition, the accumulation and translocation data are based on these twelve plants pooled into four separate samples and are log-transformed prior to statistical analyses.

1. Cadmium-imposed root growth inhibition for Wt, M10 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	51.9	25.9	9.3	($p < 0.001$)
cadmium exposure	5	1474.8	295.0	106.2	
p-line x Cd (II)	10	176.3	17.6	6.4	($p < 0.001$)
within subgroups	72	199.9	2.8		

2. Cadmium-imposed root growth inhibition for Wt, H2 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	110.9	55.4	42.4	($p < 0.001$)
cadmium exposure	4	35.5	8.9	6.8	
p-line x Cd (II)	8	80.2	10.0	7.7	($p < 0.001$)
within subgroups	60	78.4	1.3		

3. Shoot-root ratio for cadmium exposure for Wt, M10 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.37	0.19	26.25	$p < 0.001$
cadmium exposure	4	1.27	0.32	44.76	
p-line x Cd (II)	8	0.24	0.03	4.31	$p < 0.001$
within subgroups	45	0.32	0.01		

4. Shoot-root ratio for cadmium exposure for Wt, H2 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.39	0.19	14.81	$p < 0.001$
cadmium exposure	3	0.99	0.33	25.49	
p-line x Cd (II)	6	0.06	0.01	0.72	NS
within subgroups	48	0.62	0.01		

which raises the question to what extent the enhanced root Cd tolerance in the double transformant could have been produced by enhanced Cd translocation to the shoot. However, comparing the root growth and root Cd accumulation

Table 2 continued

5. Shoot-root ratio for zinc exposure for Wt, H2 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.11	0.05	12.91	$p < 0.001$
zinc exposure	3	0.39	0.13	31.79	
p-line x Zn (II)	6	0.19	0.03	7.90	$p < 0.001$
within subgroups	36	0.15	0.004		

6. Shoot cadmium concentration (µmol Cd/g dw) for Wt, H2 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.14	0.07	6.22	$p < 0.010$
cadmium exposure	3	23.05	7.68	684.21	
p-line x Cd (II)	6	0.03	0.005	0.41	NS
within subgroups	48	0.54	0.01		

7. Shoot zinc concentration (µmol Zn/g dw) for Wt, H2 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.03	0.02	3.73	$p < 0.05$
zinc exposure	3	35.84	11.95	2616.77	
p-line x Zn (II)	6	0.05	0.009	2.00	NS
within subgroups	36	0.16	0.004		

8. Root cadmium concentration (µmol Cd/g dw) for Wt, H2 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.13	0.06	6.69	$p < 0.010$
cadmium exposure	3	33.18	11.06	1147.45	
p-line x Cd (II)	6	0.04	0.007	0.70	NS
within subgroups	48	0.46	0.01		

9. Root zinc concentration (µmol Zn/g dw) for Wt, H2 and M+H

Source of variation	*df*	*SS*	*MS*	*F*	*p value*
plant line	2	0.06	0.03	9.83	$p < 0.001$
zinc exposure	3	39.77	13.26	4494.65	
p-line x Zn (II)	6	0.08	0.01	4.30	$p < 0.010$
within subgroups	36	0.11	0.003		

data from experiment 2 (figure 2a & figure 6a), it appears that the lower root Cd accumulation in the double transformant is by far insufficient to explain its superior tolerance to this metal, suggesting that improved sequestration within the root must be the major determinant. Due to insufficient data we can not establish whether this was also the case in experiment 1 (figure 1). There is a clearly different response of the root growth in the double mutant between these experiments, which might have something to do with the

germination and pre-culture conditions (agar versus soil). However, also in experiment 1, the difference in root Cd accumulation between the plant lines were marginal, like in experiment 2, once more suggesting that improved root-internal sequestration is overall the major determinant of enhanced root Cd tolerance in the double transformant. The nature of the synergism between the transgenes remains elusive, but the contributions of HMA4 and MT2b may be expected to consist of Cd efflux from the root cytoplasm into the cell walls (Papoyan and Kochian 2004; Mills et al., 2005), and the buffering of physiologically available Cd in the root cell cytosol, respectively (Zhou and Goldsbrough 1993). Remarkably, there was no significantly better shoot performance of the double mutant compared with wild type and the single gene transformants under toxic Cd exposure. The reason for this is elusive. Possibly, if the shoot would also possess a higher tolerance capacity, this might have been masked by a higher shoot Cd accumulation, due to enhanced translocation (figure 3 & figure 4a). Interestingly, expression of *AhHMA4* under its own promoter in *A. thaliana* resulted in a much lower shoot tolerance to Cd and Zn, due to enhanced translocation of these metals (Hanikenne et al., 2008). In case of expression of *HMA* under the 35S promoter, both higher root tolerance and shoot tolerance might be expected, because it could then function to promote the efflux of Cd from the cytosol into the cell wall in all the plant organs, instead of exclusively loading it into the xylem. On the other hand, it is not easy to envisage how *HMA* expression under the 35S promoter, albeit in combination with *MT2b* expression, such as in the present study, could lead to enhanced root to shoot translocation. However, also Verret et al, (2004) found enhanced translocation of Zn and Cd to the leaves next to enhanced Cd and Zn root tolerance, in *A. thaliana* upon ectopic *HMA4* expression under the 35S promoter, albeit without co-expression of MT2b. The precise reasons for this are unclear. In conclusion, our results suggest that gene stacking can produce stronger phenotypes, as compared with single gene transformations. However, we must admit that the Cd tolerance and Cd and Zn translocation phenotypes that we obtained for the double transformant are significant, but, except for Cd tolerance in roots, fairly inconsiderable and

by no means comparable with those in hyperaccumulators. The reasons for this might be that we did not choose the right genes although, on the other hand, it is evident that at least *HMA4* is a key gene in the hyperaccumulation syndrome (Hanikenne et al., 2008). It is also possible that more genes are required to obtain stronger phenotypes. Finally, it is likely that the phenotypes are limited due to incorrect localization of the transgene products at the tissue or organ level, owing to the use of the 35S promoter. This is clearly illustrated by comparing the results obtained by Verret et al. (2004) and by us in the present study with those of Hanikenne et al. (2008). This comparison suggests that the native *AhHMA4* promoter provides the correct tissue localization in *A. thaliana*, and produces a much stronger translocation phenotype than the 35S promoter does. In addition, there is evidence that cis-regulatory changes, next to gene multiplication, can be responsible for *HMA4* overexpression in natural hyperaccumulators (Hanikenne et al., 2008), which suggests that the gene promoters of these plants can provide a good alternative to the frequently used 35 S promoter.

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Chapter 6

General Discussion

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Screenings, followed by classical breeding and selection, as well as genetic engineering are the two approaches addressed within this thesis. Extraction of metals, especially Cd, Zn and to a lesser extent As, from moderately contaminated soil by means of *B. napus* seemed to be a promising application, since this species is widely used as an oil crop (with bio-diesel potential), easy to be adopted in existing farming systems, and with a high biomass production. The 77 accessions of *Brassica napus* (from all over the world) that were screened on hydroponics in this study showed a relatively low natural variation concerning metal uptake and transport to the shoot in comparison to other species screened on these characteristics, i.e. willow and poplar (Unterbrunner et al., 2007). Furthermore, it appeared that screening on hydroponics, to try to find suitable candidates accessions for field purposes, is probably not the right way to go, due to the lack of correlation between the results of the hydroponics and field experiment (this thesis, chapter 2). More striking and elegant evidence at this point was provided by another group, who found completely different Quantitative Trait Loci (QTLs) for metal uptake in Recombinant Inbred Lines (RILs) of *A. thaliana* grown on soil versus hydroponics (Ghandilyan et al., 2006).

Considering the above we decided to use contaminated soil, originating from our field location (Balen, Belgium, chapter 2) in our second greenhouse experiment. By establishing F2 crosses between promising parent accessions of *B.napus*, and growing them on homogenized contaminated soil from the field, we screened for transgressive segregation of shoot Cd concentration and shoot Cd content among a number of promising F2 families. The plant Cd uptake from this soil was significantly correlated with that of Zn which was present at 390 ± 72 mg kg-1 dry soil. This correlation is probably due to uptake or storage by common transporters, by virtue of the chemical and physical similarity of both metals (Lombi et al., 2001; Zhao et al., 2002) At higher soil Zn concentrations, Cd uptake was reduced in *T. caerulescens*, probably through competition for common transporters (Roosens et al., 2003). It was argued by Chaney et al. (2005) that a Zn/Cd ratio > 100 in soils (often found in mine wastes and most other sources of Cd and Zn) causes Zn

hyperaccumulation to reach phytotoxic levels and limits yields of most plant species before much Cd can be accumulated in shoots. Nearly all soils which require Cd remediation to protect human health are rice soils with Zn as well as Cd contamination (Chaney et al., 2004). Thus any plant/crop species used for Cd phytoextraction must be able to tolerate high Zn concentrations inside the plant body. However, some accessions of *Thlaspi caerulescens*, i.e. those from the region around the village of Ganges (Southern France), exhibit a preference for Cd accumulation over Zn accumulation and maintain high rates of Cd uptake even at external Zn/Cd concentration ratios > 100 (Zhao et al., 2002). Their use in Cd phyto-extraction practices has been advocated before (Chaney et al., 2005). However, agricultural practices for this species have not been developed yet, and additional economic value is lacking.

Although the genetic make-up of *B. napus* L., an allo-tetraploid, did not leave room for much hope, the results were not disappointing. The parents significantly differed from each other in their shoot Cd concentrations and transgression analysis showed one case of significant transgression regarding this parameter among five F2 inter-accession crosses (chapter 3). With respect to the total shoot Cd content, even three out of five inter-crosses showed significant transgression, albeit mainly due to the transgressive segregation of shoot biomass, which may be of limited value for further long-term selection for phyto-extraction capacity. However, the type of genetic variation for shoot Cd concentration among the accessions tested apparently can lead to transgressive segregation, which offers opportunities to obtain, through directional selection, shoot Cd concentrations higher than the maximum ones represented among the accessions through screening. However, it seems unlikely that hyperaccumulator-like foliar Cd concentrations can be obtained through breeding and selection.

A potentially more successful alternative for breeding and selection is genetic engineering. In this study we tested the effects of heterologous expression of two metal homeostasis genes from *A. thaliana*, *AtMT2b* and *AtHMA4*. To this end we chose tobacco as a receiver species, because it is more easily and quickly to transform than *B. napus*. Higher plant metallothioneins are

believed to function in Cu homeostasis and tolerance (Murphy and Taiz 1995; van Hoof et al., 2001; Guo et al., 2003; Roosens et al., 2004; Guo et al., 2008) and they have been suggested to function in non-essential metal detoxification too (Merrifield et al., 2004; Merrifield et al., 2006; Guo et al., 2008). HMA4 is a plasmamembrane-localized1b-type heavy metal transporting ATPase which is mainly expressed in the xylem parenchyma cells. It functions in Zn xylem loading in *A. thaliana*, probably through effluxing Zn from the parenchyma cells (Hussain et al., 2004). Enhanced expression of this gene in the related hyperaccumulator, *A. halleri*, appeared to be essential for the high rate of root to shoot translocation of Zn, as well as for the high levels of Zn and Cd tolerance in this species (Hanikenne et al., 2008). In an interspecific segregating backcross between *A. halleri* and the non-hyperaccumulator *A. lyrata*, *HMA4* co-localized with a major QTL for both Cd tolerance and Zn tolerance (Courbot et al., 2007).

We chose to test *AtMT2b* expressing tobacco plants for As(III) uptake, translocation and tolerance, because in earlier research MT over-expression did not necessarily increase shoot Cd concentration (Lugon-Moulin et al., 2004). In one of our own pilot studies (data not shown), we neither found any effect on Cd accumulation. Contrary to our expectations, *AtMT2b* expression decreased As(III) tolerance and enhanced arsenic root to shoot translocation in tobacco. Bleeker et al. (Bleeker et al., 2006) found that As(III)-PC or As(III)-GS_3 complex formation is required for vacuolar arsenic sequestration in root cells. In this way, *MT2b* expression in the rhizodermal or root cortical cells could interfere with vacuolar sequestration through binding of As(III), keeping As(III) in the cytosol and available for radial transport across the root and subsequent loading into the xylem, which might explain the translocation phenotype in the highest *AtMT2b*-expressing transformant line. Alternatively, the translocation phenotype might as well result from an enhanced bidirectional transport via the phloem. For example, transport of Cd via the phloem, probably as Cd-PC complex, has been demonstrated in *Brassica juncea* and *A. thaliana* (Gong et al., 2003; Chen et al., 2006; Mendoza-Cózatl et al., 2008). Additional research should address the question

of whether there are As(III)-MT complexes present in the cytosol of root cells and how these complexes contribute to xylem or phloem loading.

In an attempt to further explore the possibilities of genetic engineering for phyto- extraction purposes, expression of multiple genes was the next step. The aim was to combine a high biomass producing plant like tobacco, with an increased tolerance to and transport of Cd and Zn to the shoots. We successfully expressed *AtMT2b* and *AtHMA4* simultaneously in tobacco and found an increased Cd tolerance and an enhanced root to shoot transport of Cd and Zn. The higher Cd tolerance could be due to a synergistic effect between the two transgenes, because either transgene alone did not render similar tolerance to Cd, which corroborates with findings of Lugon-Moulin et al. (2004). MTs also seem to act as hypostatic factors, enhancing the tolerance level when other epistatic tolerance genes are also higher expressed (van Hoof et al., 2001). Any effect of MT over-expression on Cd tolerance could rely either on Cd binding or, conceivably, on enhanced protection against Cd-induced oxidative stress. In *E.coli* mutants hypersensitive to oxidative stress, due to mutations in the thioredoxin and glutathione reduction pathways, MTs were protecting against Cd toxicity (Moreau et al., 2008). A role for MTs as anti-oxidants has also been shown in *Arabidopsis* over-expressing the MT1 gene from *Brassica rapa*, effectively detoxifying Cd and H_2O_2 (Kim et al., 2007). No Zn tolerance phenotype was found for our double transformant, although tolerance-correlated enhanced MT2b expression has been found in Zn-hypertolerant, calamine populations of the non-hyperaccumulating *Silene vulgaris* (Jack et al., 2007). AtHMA4 functions in the root to shoot translocation of Zn (Hussain et al., 2004; Sinclair et al., 2007), and high *AhHMA4* expression levels are responsible for Cd and Zn hypertolerance and the highly efficient root to shoot Zn transport and Zn hyperaccumulation in the shoot of *A. halleri* (Hanikenne et al., 2008). In *A. halleri*, in which metals are primarily detoxified and stored in the leaves, the strong contribution of HMA4 to Cd and Zn hypertolerance might primarily lie in preventing Zn and Cd to be accumulated at toxic levels in the roots. However, in our double transformants Cd root to shoot translocation was only marginally higher than

in wild type, probably because of incorrect tissue-specificity, due to the use of the 35S promoter, and is therefore probably not the major determinant of the Cd hypertolerance in this line. It seems likely that enhanced Cd efflux across the plasma membrane, into the apoplasmic space is more important here (Mills et al., 2003; Papoyan and Kochian 2004; Courbot et al., 2007). The nature of the apparent synergism between the transgenes in the double transformant remains elusive. It is conceivable that MT2b serves as a cytoplasmic Cd buffer, which does not produce much tolerance by itself because of rapid saturability. However, it could keep a bigger part of the cellular Cd available for efflux via HMA4, the latter regenerating the buffering capacity.

Although our experiments confirm the potential of gene stacking in engineering suitable phytoremediator plants, the phenotypes that we obtained for Zn and Cd translocation in the double transformant are still inconsiderable in comparison with natural hyperaccumulators of these metals. The reasons for this are obscure, but may be related to the use of the 35S promoter (see above), which could strongly restrict the potential impact of HMA4 over-expression on metal root to shoot translocation. This problem might be overcome by using the native gene promoters from natural hyperaccumulators in future experiments. In addition, stacking more genes could further contribute to higher levels of accumulation in the shoot. Strong over-expression of HMA4 in *A. thaliana*, in the native tissue, which is the xylem parenchyma, can create a metal deficiency response in the roots, involving enhanced expression of metal uptake transporters and thus, enhanced uptake (Hanikenne et al., 2008). However, in the most effectively accumulating hyperaccumulator accessions, the root metal concentrations may greatly exceed those in non-accumulators, even at fairly low metal exposure rates, which indicates that the enhanced uptake rates in natural hyperaccumulators are not driven by efficient translocation to the shoot (Richau and Schat 2009), which in turn suggests that genes encoding uptake transporters, at least when expressed in the right tissues, are certainly candidates in gene stacking approaches. Also genes producing enhanced metal tolerance, e.g. vacuolar transporters such as MTP1 (Drager et al., 2004; Willems et al., 2007), should be added,

to cope with more strongly enhanced foliar metal influx rates. Such genes should be expressed in leaves rather than in roots, to prevent metal retention in root cell vacuoles. In summary, to engineer an efficient hyperaccumulator crop through gene stacking, the most promising way to go is to stack gene combinations that include an uptake transporter, a xylem loading transporter (HMA4), a xylem de-loading transporter (unknown), a vacuolar transporter (MTP1), and a tolerance/translocation enhancer (an MT). Preferably, these genes should be expressed under tissue-specific promoters, if possible those of natural hyperaccumulators, which can be considerably stronger than the corresponding ones in non-hyperaccumulators (Hanikenne et al., 2008).

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Summary

Exploring cadmium phytoextraction with *Brassica napus* and *Nicotiana tabacum*: breeding and selection versus genetic engineering

Successful phytoextraction of heavy metals in the field is currently limited. Although research on this topic is plentiful, breakthroughs for application in the field have only been achieved for mercury. This work attempts to shed new light on this problem by comparing two different approaches. We explored the potential for selective breeding for cadmium (Cd) accumulation in *Brassica napus*, a potentially useful candidate as a phyto-extracting crop. We also investigated the effects of heterologous expression of one or more genes involved in metal accumulation and tolerance in a high biomass yielding and easy to transform species, *Nicotiana tabacum*.

To explore the natural variation in *B. napus* concerning Cd accumulation, we compared 77 accessions from different geographic regions in hydroponics. The results demonstrate a generally low natural variation for the accumulation and root to shoot translocation of Cd. A number of accessions were selected to be tested in the field at two different locations. We observed two accessions to perform well at both locations but they did not show promising phyto-extraction potential. Based on a transgression analysis of F2 crosses between the most promising varieties, it seems that the possibilities to breed and select *B. napus* lines with significantly improved Cd phytoremediator properties are overall limited, and that genetic engineering strategies might be more successful on the short term.

Our second approach entailed the heterologous expression of one or more metal homeostasis genes in *N. tabacum*. Firstly we expressed the metallothionein 2b gene from *A. thaliana* under the 35S CMV promoter via leaf disc transformation. Earlier studies showed no obvious role in Cd homeostasis for *AtMT2b*, therefore we choose to investigate the effect of AtMT2b on arsenite uptake and transport. Our main findings here are a significantly decreased arsenite tolerance and arsenic accumulation in roots,

but an increased accumulation in shoots, while the total amount of arsenic taken up remained unchanged, suggesting that *AtMT2b* expression enhanced the arsenic root to shoot transport.

Metal uptake and translocation seem to be regulated by a complex homeostatic network, rather than by individual genes. For this reason, expressing supposedly synergistic genes together might be a rewarding strategy. Therefore, we re-transformed the single transformant *AtMT2b* tobacco T2 line with *AtHMA4*, encoding a stelar heavy metal transporting 1b-type ATPase, previously shown to function in Zn and Cd xylem loading. Cadmium and zinc (Zn) tolerance, uptake and translocation were measured in the double transformant, and compared to untransformed ('wild type') tobacco and single gene transformants. The double transformant exhibited enhanced Cd-tolerance, enhanced Cd and Zn root to shoot transport, but unaltered Zn tolerance and Cd and Zn uptake, compared with wild type. The single transformant lines did not show significant phenotypes. Our results suggest that the phenotypes of the double transformant are due to synergistic interaction between the transgenes.

The research described in this thesis suggests that the possibilities to obtain effective phytoremediator crops through breeding and selection are limited. Genetic engineering seems to be promising, particularly when using combinations of synergistic genes. However, the phenotypes that we obtained in this study were only moderate, not comparable with those of natural hyperaccumulators. One of the reasons for this is that we did not express a number of genes that contribute essentially to the natural hyperaccumulation trait. Another reason is probably that we expressed the transgenes under the 35S CMV promoter, which is expected to result in incorrect tissue-specificity patterns, potentially leading to less effective root to shoot transport or incorrect metal compartmentalization at the tissue level. Using the gene-specific promoters from natural hyperaccumulators might be a suitable strategy to overcome this problem.

Samenvatting

Cadmium phyto-extractie met *Brassica napus* en *Nicotiana tabacum*: veredeling versus genetische manipulatie

Phyto-extractie van metalen in de praktijk kent nog niet veel successen. Ondanks dat er veel onderzoek is gedaan en nog steeds gaande is, zijn echte doorbraken in het veld tot nu toe alleen bereikt voor kwik. Het werk in deze thesis probeert met behulp van twee verschillende aanpakken nieuw licht op dit onderwerp te werpen. We hebben onderzocht wat de mogelijkheden zijn van kruising en selectie voor verhoogde accumulatie van cadmium (Cd) in de bovengrondse delen van *Brassica napus* (koolzaad), een potentiele kandidaat als phyto-extractie gewas. We hebben ook onderzocht wat het effect is van expressie van 1 of meerdere genen, die betrokken zijn bij metaal accumulatie en tolerantie, in een gewas dat veel biomassa oplevert en tegelijkertijd eenvoudig genetisch te transformeren is, *Nicotiana tabacum* (tabak).

Om de natuurlijke variatie in Cd accumulatie te onderzoeken in *B. napus,* hebben we 77 accessies uit verschillende geografische gebieden met elkaar vergeleken in een hydrocultuur studie. De resulaten laten een in het algemeen lage natuurlijke variatie zien tussen de accessies voor de accumulatie en het transport van wortel naar spruit van Cd. Een aantal accessies werden geselecteerd om vervolgens getest te worden in het veld op twee verschillende locaties. Twee daarvan groeiden op beide locaties goed, maar waren niet veelbelovend met betrekking tot hun phyto-extractiecapaciteit, Gebaseerd op een transgressie-analyse van F2 kruisingen, tussen de meestbelovende varieteiten, blijkt dat de mogelijkheden tot het veredelen en selecteren van *B. napus* lijnen met significant verbeterde Cd phytoremediatie eigenschappen gelimiteerd zijn en dat genetische transformatie waarschijnlijk meer succesvol zal zijn op de korte termijn.

Onze tweede aanpak was het tot expressie brengen van 1 of meerdere genen, betrokken bij de metaalhomeostase, in *N. tabacum*. Ten eerste hebben we het metallothionine 2b gen uit *Arabidopsis thaliana* (AtMT2b) tot expressie

gebracht onder de 35S CMV promoter via de bladschijftransformatie methode. Eerder onderzoek heeft uitgewezen dat AtMT2b geen duidelijke rol speelt in Cd homeostase, daarom hebben we ervoor gekozen om het effect van AtMT2b op de opname en het transport van arseniet te onderzoeken. Onze voornaamste bevindingen waren een significante afname van de arseentolerantie en de arseenaccumulatie in de wortel, maar een toegenomen accumulatie in de spruit, terwijl de totale hoeveelheid opgenomen arseen onveranderd bleef. Dit suggereert dat de expressie van AtMT2b het transport van arseen van wortel naar spruit bevordert.

Metaalopname en -translocatie worden gereguleerd door een complex homeostatisch netwerk en niet door individuele genen. Het samen tot expressie brengen van veronderstelde synergistische genen kan een veelbelovende strategie zijn. Daarom hebben we de AtMT2b-transformant van tabak opnieuw getransformeerd met *AtHMA4*, coderend voor een P-type ATP-ase dat voornamelijk in de stele van de wortel tot expressie gebracht wordt. Eerdere studies hebben uitgewezen dat AtHMA4 een belangrijke rol speelt in het 'laden' van het xylem met zinc (Zn) en Cd. Cd- en Zn- tolerantie, -opname en -translocatie zijn gemeten in de dubbel-transformant en vergeleken met ongetransformeerde 'wild type'tabak en met één gen getransformeerde tabak ('enkeltransformanten'). De dubbeltransformant had een verbeterde Cd-tolerantie en een verbeterd Cd- en Zn-transport van wortel naar spruit, maar een onveranderde Zn-tolerantie en Cd- en Zn-opname vergeleken met het wildtype. De beide enkeltransformanten hadden geen significante fenotypen. Onze resultaten suggereren dat de fenotypen van de dubbeltransformant veroorzaakt worden door een synergistische interactie van de transgenen.

Het onderzoek beschreven in deze thesis suggereert dat de mogelijkheden tot het verkrijgen van een effectief phytoremediatiegewas beperkt zijn als we dit alleen proberen via kruising en selectie. Genetische manipulatie lijkt veelbelovend, vooral wanneer er gebruik gemaakt wordt van synergistische genen. Maar, de fenotypen die we gevonden hebben in deze studies zijn gematigd, en niet te vergelijken met die van natuurlijke 'hyperaccumulatoren' van zware metalen. Een van de redenen hiervoor kan zijn dat we niet meer

dan twee genen tot expressie hebben gebracht die essentieel zijn voor de hyperaccumulatie eigenschap. Een andere reden is mogelijkerwijs het feit dat we de transgenen tot expressie hebben gebracht onder de 35 S CMV promoter, hetgeen kan resulteren in een foutieve weefselspecificiteit van de expressie, mogelijk leidend tot minder effectief metaaltransport van wortel naar spruit of tot een foutieve metaalcompartering op weefselniveau. Het gebruik van gen-specifieke promoters afkomstig van natuurlijke hyperaccumulatoren kan een geschikte strategie zijn om deze problemen te voorkomen.

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